Immune responses against spontaneous tumors in a murine model for pancreatic adenocarcinoma

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1 INTRODUCTION

1.1 Tumor Immunity

The first trial of cancer immunotherapy can be dated back to the 19th century, when William Coley, a New York surgeon, used extracts of pyogenic bacteria to treat tumors (Coley, 1893). Coley was able to show that this nonspecific activation of the immune system could result in recovery or even in tumor regression and cure of some patients. Some decades later, it was demonstrated that tumors could be induced in mice after treatment with chemical carcinogens. These findings together with the discovery of MHC and the development of inbred mouse strains by Gorer and Snell (Gorer, 1956) made it possible to undertake the first immunological experiments with transplantable tumors. Transplanted tumors in mice exhibit a variable pattern of growth when injected into syngenic animals: Some are rejected when transplanted into syngenic hosts (regressive growth), but most tumors grow progressively and eventually kill the host.

The first demonstration that inbred mice could be immunized against transplanted tumors was in 1943. Gross and colleagues showed that intradermal immunization of mice against sarcoma protects the animals from outgrowth of the same tumor (Gross, 1943). Some years later, it was demonstrated in more detailed experiments that normal tissue of the mouse of tumor origin did not immunize against the transplanted tumor, nor did the tumor cells immunize against normal skin grafts from this mouse (Prehn and Main, 1957; Foley, 1953; Baldwin, 1955; Klein et al., 1960; Old et al., 1962). These first transplantation experiments revealed that this immunological protection against challenge with tumor cells was individually tumor specific. The unique specificity indicated that each tumor had a unique transplantation antigen and/or expressed a unique combination of shared antigens. This could not only be shown for transplanted tumors originally induced by chemical carcinogens, but also by physically carcinogens or by spontaneous tumors (Globerson and Feldman, 1964; Pasternak et al., 1964; Kripke, 1974) (Basombrio, 1970; Basombrio and Prehn, 1972). Since these protective effects cannot be shown in T cell deficient mice but can be compensated by adoptive transfer of T cells from immunized mice, it was suggested that tumors express antigenic peptides that can be recognized by tumor-specific T cells.

1.1.1 Tumor immunosurveillance

The notion that the immune system could protect the host from neoplastic disease was initially proposed by (Ehrlich, 1909) and formally introduced as the cancer immunosurveillance hypothesis nearly 50 years later by Thomas and Burnet (Thomas, 1959; Burnet, 1970): They claimed that tumors arise with similar frequency to infection with pathogens and that the immune system constantly recognizes and eliminates these tumors based on their expression of tumor antigens. During the last decades, this hypothesis has evoked strong criticism, but today more and more studies in animal models as well as in certain human diseases support the view of Thomas and Burnet that the immune system monitors and modulates tumor growth (Dunn et al., 2002; Darnell and Posner, 2003). For instance, studies using gene-targeted mice that lack the recombinase activating gene (RAG)-1 or (RAG)-2 (Shinkai et al., 1992) showed that these immunodeficient mice, which cannot rearrange lymphocyte antigen receptors and thus lack T, B and NKT cells, have enhanced susceptibility to chemically (e.g. methylencholanthrene [MCA]) induced and spontaneous tumors (Shankaran et al., 2001; Smyth et al., 2001a). Results of relevant studies on different mouse models were reviewed by Dunn *et al.* (Dunn et al., 2002; Dunn et al., 2004) and are summarized in Table 1.1. Taken together, these observations demonstrate that tumor development in mice is controlled by components of the immune system. The induction of tumor-specific immune responses results from complex interactions of antigen presenting cells (APCs), cytotoxic T cells (CTLs), T helper cells and antibody-producing B cells as well as effector cells of the innate immune system like natural killer cells (NK cells) and NKT cells which can recognize transformed cells and eradicate them.

Phenotype or depletion	Immune deficiency	Tumor susceptibility	Reference
RAG2$^{-/-}$ RAG1$^{-/-}$	Lacks T, B and NKT cells	MCA-induced sarcomas Spontaneous intestinal neoplasia	(Shankaran et al., 2001; Smyth et al., 2001a)
αβ T cell$^{-/-}$	Lacks αβ T cells	MCA-induced sarcomas	(Girardi et al., 2001; Gao et al., 2003)
γδ T cell$^{-/-}$	Lacks γδ T cells	MCA-induced sarcomas	(Girardi et al., 2003; Gao et al., 2003; Girardi et al., 2001)
Anti-NK1.1 antibody	Lacks NK and NKT cells	MCA-induced sarcomas	(Smyth et al., 2000; Smyth et al., 2001a)
Anti-asialo-GM1 antibody	Lacks NK cells, monocytes, macrophages	MCA-induced sarcomas	(Smyth et al., 2001a)
IFN-γ$^{-/-}$	Lacks IFN-γ	MCA-induced sarcomas B6:spontaneous disseminated lymphomas BALB/c : spontaneous lung adenocarcinomas	(Street et al., 2001; Street et al., 2002)

Table 1.1: Evidence supporting the tumor immunosurveillance hypothesis: Enhanced susceptibility of immunodeficient (genetically engineered or *in vi*vo depleted) mice to formation of spontaneous and chemically induced tumors (adapted from Dunn 2002)

1.1.2 Tumor antigens recognized by T cells

Any protein in the tumors cell is a potential tumor antigen (also called tumor rejection antigens, tumor-associated antigens or in the mouse system tumor-specific transplantation antigens). Peptides derived from tumor antigens can become the targets of a tumor-specific T cell response, because they are not displayed on the surface of normal cells, at least not at levels sufficient to be recognized by T cells.

Most of the murine and human tumor antigens now known have been identified by screening tumor derived cDNA libraries using tumor-reactive T cell lines and clones from cancer patients, thus identifying the gene encoding the target epitope (De Plaen et al., 1988; Lurquin et al., 1989; van der Bruggen et al., 1991; Boon, 1993; Rosenberg, 1996, summarized in van der Bruggen et al., 2002). Several other antigenic peptides have been found by peptide elution followed by mass spectrometry analysis (Cox et al., 1994; Mandelboim et al., 1994; Huang et al., 1996). An alternate technique for the identification of tumor antigens, which is not dependent on T cell recognition, is the serological analysis of recombinant cDNA expression libraries (SEREX). In this approach, diluted sera of cancer patients are used to identify circulating Immunoglobulin (Ig)G that are specific for tumor antigens (Sahin et al., 1997; Chen et al., 1997; Jäger et al., 1998; Krackhardt et al., 2002). The first antigens identified with this method were two melanoma antigens (melanoma antigen 1 [MAGE-1] and tyrosinase) and the esophageal cancer-associated antigen NY-ESO-1. MAGE-1 and tyrosinase were originally identified by cloning the epitopes recognized by T cells, and NY-ESO was subsequently shown to be reactive with T cells. (summarized in Greten and Jaffee, 1999 and Rosenberg, 1999).
In addition, potential target antigens have been defined by gene expression profiling searching for overexpressed gene products in neoplastic cells compared with normal tissues. Microarray-based studies of gene expression in a large number of tumor types have repeatedly revealed novel features of human cancer and have led to the identification of a very large number of potential tumor antigens (summarized in Mohr et al., 2002).

Different categories of tumor antigens can be distinguished and examples of each of these are given in Table 1.2. The first category consists of antigens that are strictly tumor-specific. These antigens are the result of point mutations or gene-rearrangements, which often arise as part of the process of oncogenesis. The best example for this might the cyclin-dependent kinase 4 (CDK4)-R24C antigen, the result of a mutation in CDK4. The mutation occurs within the antigenic epitope recognized by $CD8^+$ T cells. In addition, this mutation prevents binding of a CDK4 inhibitor, thereby disrupting cell-cycle regulation (Wolfel et al., 1995). Another example is the mutated tumor suppressors genes p53 (Theobald et al., 1995). Mutations of p53 are found in 60-70% of all human cancers (Greenblatt et al., 1994).

The second category includes tumor specific antigens such as MAGE or NY-ESO. Tumor specific antigens are silent in all normal adult tissues except for male germ cells, which do not

express major histocompatibility complex (MHC) molecules and therefore cannot present peptides from these molecules to T cells. These genes are expressed in a significant proportion of tumors, including melanomas, lung tumors, head and neck tumors, and bladder carcinomas.

Tissue-specific differentiation antigens belong to the third category of tumor antigens. These antigens are encoded by genes that are only expressed in particular types of tissues. For example, tyrosinase (Brichard et al., 1993; Robbins et al., 1994; Wolfel et al., 1994; Kittlesen et al., 1998), MART-1/Melan-A (Coulie et al., 1994; Kawakami et al., 1994a; Kawakami et al., 1994b) and gp100 (Cox et al., 1994; Bakker et al., 1994) are presented on normal melanocytes as well as on melanomas.

The fourth category is comprised of antigens that are strongly overexpressed in tumor cells compared with their normal counterparts. A typical example for this is HER/2-neu, which is a receptor tyrosine kinase homologous to the epidermal growth factor receptor (Fisk et al., 1995). This receptor is overexpressed in many adenocarcinomas, including breast and ovarian cancer.

The fifth category of tumor antigens includes molecules that display abnormal tumor-specific posttranscriptional modifications. An example is the aberrant glycosylated mucin, MUC1, which is expressed by a number of tumors, including breast and pancreatic cancers (Girling et al., 1989; Finn et al., 1995).

Antigens derived from oncogenic viruses represent the sixth category of potential tumor antigens. These viral antigens play a critical role in the oncogenic process and, because they are foreign, can induce T cell responses. Examples are the oncoproteins E6 and E7 of human papilloma virus 16 (HPV16), which is present in most cervical carcinomas (Wu, 1994).
(summarized in Boon et al., 1997; Greten and Jaffee, 1999; Rosenberg, 1999; Janeway and Travers, 2001)

While most of the focus in cancer immunology is on $CD8^+$ cytotoxic T lymphocyte responses, there is more and more evidence that $CD4^+$ cells also play an important role in tumor immunity (Pardoll and Topalian, 1998; Toes et al., 1999; Wang, 2001). $CD4^+$ cells can promote cellular and humoral immune responses and are essential for the priming and

persistance of $CD8^+$ T cell responses. Tyrosinase was the first MHC class II restricted antigen that was detected in some melanoma patients (Topalian et al., 1994; Topalian et al., 1996). So far, only few $CD4^+$ epitopes have been identified for several other tumor antigens such as MART-1/ Melan-A (Zarour et al., 2000a), MAGE-1 (Chaux et al., 2001), MAGE-3 (Manici et al., 1999; Chaux et al., 1999) or NY-ESO (Jager et al., 2000; Zeng et al., 2000; Zeng et al., 2001; Zarour et al., 2000b; Zarour et al., 2002) due to the lack of effective methods for identifiying MHC class II restricted tumor antigens.

Category of antigen	Antigen
Tumor-specific mutated oncogene or tumor suppressor	CDK-4 β-Catenin CASP-8 p53 K-ras
Germ cell	MAGE-1 MAGE-3 NY-ESO CEA
Differentiation	Tyrosinase MART-1/Melan-A gp100
Overexpressed antigens	HER-2/neu
Abnormal post-translational modification	MUC1
Oncoviral protein	E6 and E7 (HPV16)

Tabel 1.2. Potential origins of tumor antigens

1.1.3 Effector mechanisms in cancer immunity

1.1.3.1 Adapted effector mechanisms in cancer immunity

Several preclinical and clinical studies have demonstrated that activation of both $CD4^+$ and $CD8^+$ T cells is critical for generating the most potent antitumor immune responses (Marchand et al., 1995; Rosenberg et al., 1998; Jager et al., 1999). $CD8^+$ CTL cells are able to lyse tumor cells directly upon recognition of peptide-MHC-class I complexes expressed by the tumor. "Professional" APCs like dendritic cells (DCs) can initiate antigen-specific T and B cell responses by capturing the antigens that are secreted or shed by tumor cells or after cell lysis. As professional APCs, DCs are the most powerful stimulators of naïve T cells. Immature DCs take up and process antigens (Ridge et al., 1996; Schoenberger et al., 1998; Bennett et al., 1998). Encountering inflammatory mediators or interaction with $CD4^+$ cells via CD40 ligand (CD40L)-CD40 interaction leads to DC maturation. Mature DCs down-regulate their antigen uptake and processing machinery, express CD83 and up-regulate MHC and costimulatory molecules such as CD80 and CD86 (B7.1 and B7.2) (Banchereau and Steinman, 1998; Fong and Engleman, 2000; Schuler et al., 2003). Processing and presentation of tumor antigens by major histocompatibility complex (MHC) class I and class II molecules on a single DC can enable priming and activation of both $CD4^+$ and $CD8^+$ T cells. The ability of APCs to present endocytosed tumor antigens not only to $CD4^+$, but also to $CD8^+$ T cells is called "cross priming" (Huang et al., 1994; Albert et al., 1998). $CD4^+$ cells can be divided into T Helper (TH)1 and TH2 cells based on their cytokine secretion profile (Morel and Oriss, 1998). $CD4^+$ TH1 and TH2 cells promote cellular and humoral immune responses, respectively. CD4 TH1 cells are essential in the maintenance of $CD8^+$ CTL effector functions. Costimulation occurs via $CD4^+$ T cell derived cytokines (e.g. classical TH1 cytokines such as Interleukin (IL)-2 and Interferon (IFN)-γ or classical TH2 cytokines such as IL-4) and also through the APC itself *via* CD40-CD40L or B7-CD28 interactions. In addition, memory $CD4^+$ T cells and $CD8^+$ T cells play a critical role in maintaining the protective immunity (Swain et al., 1991; Mackay, 1993). (summarized in Greten and Jaffee, 1999 and in Smyth et al., 2001b and shown in Figure 1.1).

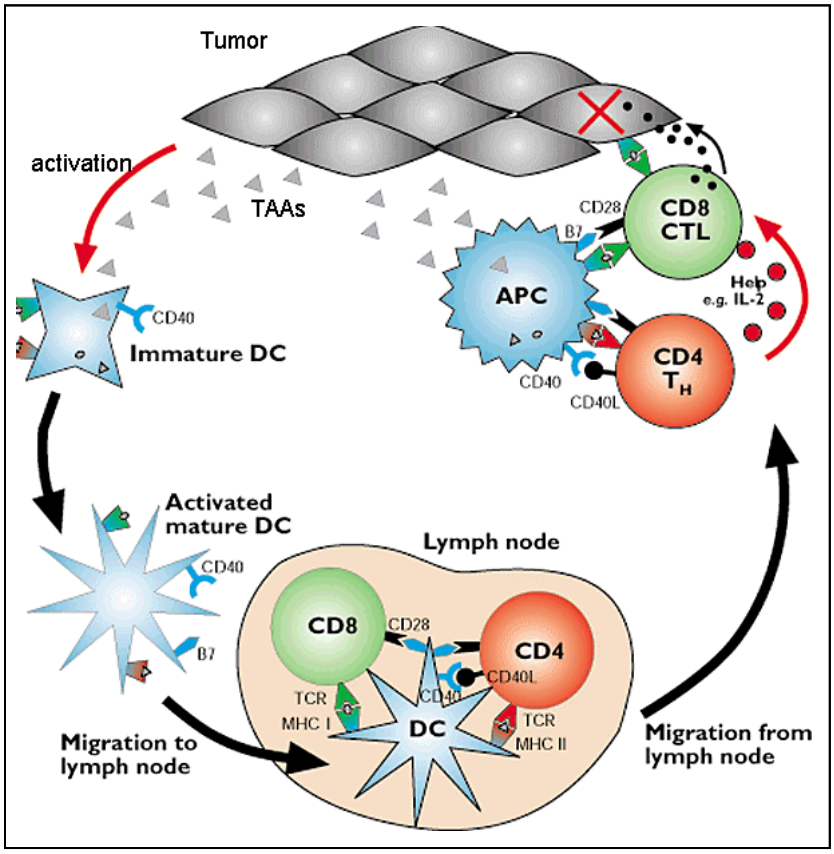

Figure 1.1: Adaptive immune response to tumor-derived antigens. The capture and presentation of tumor antigens (TAAS) by "professional" APCs like DCs is one of the earliest steps towards mounting an adaptive immune response against tumors. APCs are additionally activated by tumor cell derived signals like certain cytokines. In combination with costimulation (e.g. B7-CD28 interaction), activated APCs carry tumor antigens to the lymph node where the tumor antigen-derived peptides are presented via MHC class II molecules to $CD4^+$ and MHC class I molecules to $CD8^+$ lymphocytes. Activated $CD4^+$ cells can subsequently express CD40L, which, in turn, further stimulates CD40-expressing APCs. B cells (not shown here) are also involved in anti-tumor immune response. Tumor antigen-specific lymphocytes develop into activated effector cells – $CD4^+$ helper T cells and $CD8^+$ CTLs – which possess now the ability to migrate into the tissue to mount together with local APCs an effective response against the developing tumor (adapted from Smyth 2001).

1.1.3.2 Innate effector mechanisms in cancer immunity

NK cells can spontaneously kill MHC class I deficient tumor cells (Hoglund et al., 1988; Whiteside and Herberman, 1995). They mediate cytotoxicity via perforin and produce a variety of cytokines like IFN-γ and are highly responsive to many cytokines, including IL-2, IL-12, IL-15 and IFNs. NK cells express a set of functionally diverse receptors on their surface: As an important actor in antibody-dependent cell mediated cytotoxicity (ADCC), NK cells recognize via their FcγRIII receptor IgG antibody coated cells (Ravetch and Perussia, 1989; Lanier et al., 1989; Perussia et al., 1984). NK cells also display a subset of inhibitory receptors (e.g. KIR and Ly49) (Colonna and Samaridis, 1995; Mason et al., 1995; Stoneman et al., 1995), which are specific for MHC class I. NKG2D has been defined as an activating receptor for NK cells (Vivier et al., 2002; Jamieson et al., 2002). This receptor binds to the so called MHC class I chain related (MIC) glycoproteins, which are low or not expressed on normal adult tissue but are induced by cellular stress (Diefenbach et al., 2000; Cerwenka et al., 2000). NKG2D plays a key role in immune responses, including those against tumors (Diefenbach et al., 2001; Cerwenka et al., 2001) and has been demonstrated as a primary cytotoxicity receptor on mouse NK cells (Hayakawa et al., 2002).

NKT cells, a subset of T cells that express NK cell markers such as NK1.1 (Godfrey et al., 2000) have shown to exhibit the capacity to regulate both CTL and NK cell anti tumor activity (Smyth et al., 2000). NKT cells have a restricted TCR repertoire and recognize the nonclassical MHC class I protein CD1d in the context of glycolipids. Several studies have demonstrated the importance of NKT cells in immune regulation and in the prevention of autoimmune diseases in mice (Hong et al., 2001; Sharif et al., 2001; Miyamoto et al., 2001), which supports the idea that NKT cells can act as suppressor cells. Unlike other regulatory T cells, NKT have also been implicated as effectors in immune responses to some pathogens. NKT cells express a variety of cell-death-inducing effector molecules and have been demonstrated to kill tumor target cells *in vitro*. In addition, NKT cells rapidly secrete large amounts of both pro-inflammatory T helper 1 (TH1) cytokines such as IFN-γ, and anti-inflammatory TH2 cytokines, such as IL-4 and IL-10, bridging the innate and adaptive immune system (summarized in Smyth et al., 2002).

1.2 Mechanisms of tumor escape

Despite an active and apparently normal immune response by a healthy immune system, tumor cells can grow, invade and metastasize in the host (Pardoll, 2003). Tumor cells can escape or fail to elicit anti-tumor immune responses by various mechanisms. They are phenotypically and genetically less stable than normal cells and can rapidly change to escape immune destruction.

1.2.1 Changes in expression of MHC class I molecules

Some tumors lose or down-regulate the expression of MHC class I molecules, perhaps through immunoselection by T cells specific for a peptide presented by that MHC class I molecule. Tumor cells can use multiple mechanisms to partially or completely down-regulate the expression of MHC class I antigens. A variety of altered Human Leukocyte antigen (HLA) phenotypes have been defined in human tumors, including HLA total loss, HLA haplotype loss, HLA-specific locus down-regulation, HLA allelic losses, and a combination of these phenotypes . Decreased or absent MHC class I expression has shown to be associated with invasive and metastatic lesions (Garrido et al., 1997; Garrido and Algarra, 2001). Total loss of MHC class I expression is not uncommon in many tumors, including melanomas, colorectal and breast carcinoma and prostate adenocarcinoma (Algarra et al., 1997; Cabrera et al., 1996). In other cancers, such changes may be due to mutations in the gene coding for β2-microglobulin (Wang et al., 1993; Hicklin et al., 1998).When a tumor loses expression of its MHC class I molecules, it can no longer be recognized by cytotoxic T cells, but it might become susceptible to NK cell lysis (Porgador et al., 1997). One explanation why MHC class I negative tumor cell variants continue to grow and are not destroyed by NK cells might be the concomitant loss or down-regulation of the expression of ligands (e.g. MIC) for NK cell activation receptors (Garrido and Algarra, 2001). These mechanisms for tumor escape from host immunity may occur especially in cancer cells that cannot lose their tumor specific antigen because its expression is required for the maintenance of the malignant phenotype of the tumor cell.

1.2.2 Antigenic loss or down-regulation

Another tumor escape mechanism is the loss or down-regulation of the expression of a CTL-recognized target antigen (Urban et al., 1982; Uyttenhove et al., 1983; Ward et al., 1990), which can occur independently of MHC class I down-regulation. In the mouse system it was already demonstrated in the 1960s that loss of immunogenicity of tumors can be the result of successive transplantations, and that tumors isolated from mice originally transplanted with immunogenic tumors frequently grow with increased malignancy (Pasternak et al., 1964; Graffi et al., 1964). Also in human cancers decreased expression of melanoma-melanocyte differentiation antigens such as gp100, MART-1 and tyrosinase have been described to be associated with disease progression (de Vries et al., 1997). The mechanisms that control the down-regulation of tumor antigens might be explained by a suggested epitope immunodominance: Antigen-loss variants within a tumor are protected from immune pressure, because the parental tumor cells carry the immunodominant epitope and are therefore the ones that are noticed and eliminated by the immune system of the host (Schreiber et al., 2002).

1.2.3 Production of inhibitory cytokines by the tumor

Another way by which tumors evade rejection is the secretion of immunosuppressive cytokines. Transforming growth factor (TGF)-β and IL-10, which have been shown to be produced by a number of tumors of different tissues origins, can suppress inflammatory T cells and cell-mediated immunity and can compromise development and activity of DCs, respectively (Chen et al., 1994; Chen et al., 2001; Tada et al., 1991; Inge et al., 1992; Elgert et al., 1998; Gorelik and Flavell, 2001).

1.2.4 Suppression of anti-tumor immunity by immunoregulatory T cells

The presence of regulatory $CD4^+CD25^+$T cells within the tumor has also been shown to play a role in dampening tumor-specific immune responses (Liyanage et al., 2002; Woo et al., 2002; Woo et al., 2001). Depletion of $CD4^+CD25^+$T cells, which have been shown to be crucially involved in the prevention of autoimmune diseases (Shevach, 2000; Sakaguchi et al., 2001),

can abrogate immunological unresponsiveness to syngenic tumors *in vivo*, resulting in spontaneous tumor-specific CTL and NK cell cytotoxicity (Shimizu et al., 1999; Onizuka et al., 1999). In addition to $CD4^+CD25^+$T cells, IL-13-secreting NKT cells can exert inhibitory effects on tumor immunity.

1.2.5 Defective death receptor signaling

Death receptor ligands such as Fas ligand and TRAIL play also a role in tumor immunosurveillance. Defective death receptor signaling at multiple sites represents a mechanism that may contribute to the survival and proliferation of tumor cells, on the one hand, by rendering tumor cells resistant to death receptor-mediated apoptosis (i.e. programmed cell death) (Davidson et al., 1998; Takeda et al., 2002; Takeda et al., 2001; Straus et al., 2001) and on the other hand, by inducing activation-induced cell death (AICD) of tumor-reactive T cells (Strand et al., 1996; Walker et al., 1998; Gastman et al., 1999).

1.2.6 Tumor stroma and other local factors

Another factor that may contribute to tumor development and progression may result from the localization of a tumor; thus, the inability of the circulating T cells to reach the tumor site leads to ignorance to the presence of the tumor (Ochsenbein et al., 1999; Ochsenbein et al., 2001). For instance, the so called stromal barrier may prevent the immunological destruction of tumor cell. Tumor cells become embedded in a matirx non-malignant tissue consisting of vessels, sessile and migratory cells, and extracellular matrix, which together are termed “tumor stroma”. This stroma seems to play a complex role in tumor growth (Singh et al., 1992; Ibe et al., 2001). Furthermore, most tumors seem to grow in a non-inflammatory microenvironment. This lack of costimulatory molecules (e.g. B7.1-CD80, B7.2-CD86 and CD40 ligand) by tumor cells may lead to suboptimal activation of NK cells and T cell anergy (Schwartz, 1990).

1.3 Cancer Vaccines

Different vaccination approaches are being evaluated in clinical trials in efforts to induce host immune responses against a variety of solid tumors.

1.3.1 Whole cell vaccines

One vaccination approach is the application of whole cell vaccines. Cell-based vaccines consisting of irradiated melanoma cell lines administered with adjuvants such as Bacillus Calmette-Guerin (BCG) have been tested since the 1970s with small but significant success against melanoma (Hsueh et al., 1998; Mitchell, 2002; Sondak et al., 2002). Furthermore, genes encoding costimulatory molecules, like the B7 family or genes encoding cytokines (Golumbek et al., 1991; Gansbacher et al., 1990b; Gansbacher et al., 1990a; Asher et al., 1991; Hock et al., 1991; Porgador et al., 1992; Blankenstein et al., 1991), which can be further enhanced by the combination of both, cytokine and B7 expression (Azuma et al., 1993; Chen et al., 1992; Baskar et al., 1993; Townsend and Allison, 1993; Cayeux et al., 1995) were transferred into tumor cell vaccines to enhance the immunogenicitiy of tumor cells. Compared to other cytokines, Granulocyte–Macrophage-Colony-Stimulating Factor (GM-CSF) appears most effective. GM-CSF has the ability to recruit granulocytes, macrophages and APCs such as DCs, which could enhance cross-presentation of tumor antigens. Moreover, GM-CSF has also been shown to upregulate CD1 on APCs, suggesting a possible interaction with NKT cells (Sato et al., 1999; Dranoff, 2002). Tumor-cell vaccination using tumor cells transfected with GM-CSF have led to promising results not only in experiments with rodents (Dranoff et al., 1993; Disis et al., 1996; Chiodoni et al., 1999), but also in clinical studies (Jaffee et al., 2001; Jaffee et al., 1998; Simons et al., 1999; Salgia et al., 2003). A number of other tumor cell vaccines expressing IL-2, IL-4 and B-7.1 are currently being tested in clinical trials (Maio et al., 2002; Antonia et al., 2002).

1.3.2 Peptide based vaccines

The identification of tumor antigens provided the basis for the establishment of peptide based vaccines. The best-studied clinical model of peptide vaccination is malignant melanoma. It

was shown that the use of native peptides for immunization is often insufficient to generate reactive T cells and clinical responses in most patients. Rosenberg and colleagues for instance evaluated vaccination with native gp100 peptide and found that it produced only low levels of T cell reactivity in immunized patients, whereas an epitope-enhanced gp100 peptide (modified amino acid sequence to enhance e.g. MHC molecule binding) generated strong T cell reactivity. However, only one single objective clinical response was reported. Combination of the epitope-enhanced gp100 with high-dose IL-2 treatment resulted in an anti-tumor response in nearly half of all patients (Rosenberg et al., 1998). Further clinical approaches (Smith et al., 2003; Lee et al., 2001; Schaed et al., 2002; Marchand et al., 2003) used adjuvants such as incomplete Freund`s adjuvant (IFA), cytokines and costimulatory molecules to enhance immunogenicity of the peptide vaccination. In a new trial, blockade of CTL-associated antigen (CTLA)-4 in combination with epitope-enhanced gp100 showed promising results in melanoma patients (Phan et al., 2003). Although vaccines based on tumor antigens are, in principle, the ideal approach to T cell-mediated cancer immunotherapy, the disadvantage of a peptide vaccine is that its immunogenecity is restricted. Including a range of tumor antigens in a tumor vaccine might be an effective approach.

1.3.3 Heat-shock proteins

The use of heat-shock proteins (HSPs) isolated from tumor cells represents a novel experimental approach to tumor vaccination. HSPs are found in most cells where they act as intracellular chaperones of antigenic peptides. There is evidence for receptors on professional APCs that take up certain HSPs together with any bound peptide. It is postulated that HSPs induce a specific CTL response by introducing antigens into the MHC class I pathway. Immunizations with tumor-derived HSPs such as the HSP96, generated tumor-specific immunity in mice (Tamura et al., 1997; Blachere et al., 1997). HSP96 has already been tested successful in clinical trials (Belli et al., 2002; Marchand et al., 1995).

1.3.4 Adoptive transfer of cytotoxic T cells

Another approach of immunotherapy for cancer is the isolation of antigen-specific cells, their *ex vivo* expansion and generation, and subsequent autologous administration. The generation of T cell clones specific for tumor antigens such as MART1/Melan-A and gp100 and their effective transfer have been shown in several recent studies. The obvious disadvantage of this approach is again the required presence of defined tumor antigens (Sing et al., 1997; Rosenberg et al., 1994; Yee et al., 2002; Dudley et al., 2002; Overwijk et al., 2003) and furthermore that the *ex vivo* generation of such T cell populations is labor-intensive and time consuming.

1.3.5 CpG Oligonucelotides

Nonspecific immune adjuvant strategies such as incorporating bacterial unmethylated ssDNA sequences, so called cytosine-phosphorothioate-guanine (CpG) containing oligodeoxy-nucleotides (ODN), in the vaccine formulation have been shown to be potent in inducing anti-tumor immune response in several studies. CpGs are the ligands to toll-like receptor (TLR)-9. Signaling through TLRs can produce effects in DCs similar to CD40 ligation (Krieg, 2002; Krieg, 2003). CpGs are quite effective in the induction on an anti-tumor T cell response in a variety of mouse models (Sandler et al., 2003; Hartmann et al., 2000; Brunner et al., 2000; Weiner et al., 1997; Kawarada et al., 2001; Davila et al., 2003; Garbi et al., 2004), but so far, only one clinical trial of treating melanoma with CpGs has been reported (van Ojik et al., 2002).

1.3.6 Recombinant viral vectors and DNA Vaccines

Another approach is using recombinant viruses such as vaccinia, adenovirus, fowlpox and avipox virus to express tumor antigens. Several trials reported the induction of weak cellular immune responses against the particular tumor antigen but also the induction of antiviral neutralizing antibodies (Marshall et al., 2000) (Zhu et al., 2000; Restifo et al., 1995; McCabe

et al., 1995; Irvine et al., 1995). The problem of the immunodominance of viral antigens over tumor antigens still remains to be solved.

DNA-based vaccines, which were successfully used to induce antibody and cellular immune responses in mice (Tang et al., 1992; Ulmer et al., 1993; Condon et al., 1996), represent another approach, but have not yet shown much promise for antitumor vaccination in clinical trials (Timmerman et al., 2002; Conry et al., 2002). To enhance the efficacy of DNA-vaccines, so-called prime-boost vaccination strategies were established (Ramshaw and Ramsay, 2000). It seems that priming with naked DNA, followed by boosting with the same antigen delivered through a viral vector, is particularly effective in amplifying immune responses. Especially against infectious diseases these heterologous prime-boost immunization approaches led to promising results (McConkey et al., 2003; Hanke et al., 2002) and have therefore clear relevance for cancer (Duenas-Carrera, 2004).

1.3.7 Dendritic-cell-based vaccines

As professional APCs, dendritic cells (DCs) are the most powerful stimulators of naïve T cells. The use of antigen-pulsed autologous DCs to stimulate therapeutically useful cytotoxic T cell responses has been developed in experimental models. In these experiments, DCs were loaded *in vitro* with protein (Paglia et al., 1996), peptide (Zitvogel et al., 1996; Mayordomo et al., 1996) or tumor cell extracts (Ashley et al., 1997), were fused with whole tumor cells (Gong et al., 1997), or were transduced *ex vivo* with viral vectors or transfected with tumor cell mRNA (Specht et al., 1997; Song et al., 1997). In the first clinical trial patients with B cell lymphoma were treated with protein-pulsed DCs (Hsu et al., 1996). Further human studies with promising results were carried out with melanoma (Nestle et al., 2001; Nestle et al., 1998; Mackensen et al., 2000; Banchereau et al., 2001; Schuler-Thurner et al., 2002) gastrointestinal malignancies (Sadanaga et al., 2001; Stift et al., 2003), renal (Su et al., 2003) and prostate cancer (Fong et al., 2001).

In summary, there are three steps that are required for an effective immunotherapy for cancer: There must be sufficient numbers of tumor-reactive lymphocytes in the host, these lymphocytes must home and survive at the site of cancer, and once there, they must be able to destroy the tumor cells (Rosenberg, 2004).

1.4 Mouse models for cancer

Experiments in mice are in most cases performed by injecting fast growing tumors that have already been analyzed in transplantation experiments several times. This allows performing experiments under well standardized conditions concerning the minimal lethal dose, the latency and the growth in syngenic recipients. However, these transplantation experiments do not recapitulate the human disease in any way. Typically, transplanted tumors are inoculated subcutaneously or intravenously and therefore do not grow in the anatomically appropriate site. As a consequence, the mouse model does not mimic the organ-specific physiology characteristics of the tumor and the immune system of the mouse is not exposed to the tumor in a manner comparable to that of naturally occurring tumors in patients. Spontaneous human tumors usually develop through a gradual series of cellular changes from pre-malignant to malignant pathologies, whereas transplantable tumors generally progress very rapidly upon inoculation and the immune system of the mouse is therefore abruptly confronted with the tumor. Furthermore, vaccination against “artificial” tumor antigens only expressed by the transplanted tumor may be totally foreign for the mouse. In this case, the induction of a protective immunity against the transplanted tumor does not have to overcome tolerance. Most human cancer antigens are normal, non-mutated differentiation molecules or non-mutated proteins found only in tumor or germ cells and because of that, the human immune system seems to be more tolerant of these tumor antigens than the mouse system is of allogens. Human cancer can be imitated in the mouse by overexpressing a tumor antigen in transplanted tumors that is also expressed at low levels by a few normal tissues. In this situation the vaccine has to break tolerance, but again the short duration of the experiment is not mimicking the natural situation in a cancer patient and vaccination consequences like induction of autoimmunity can not be evaluated. Another disadvantage of transplantable murine tumors is that they are normally not spontaneously metastatic, so that vaccine strategies developed using these mouse models are not particularly relevant for human cancers, whereupon disseminating metastatic disease is frequently the predominant cause of death in most patients (summarized in Lollini and Forni, 2002; Ostrand-Rosenberg, 2004; Rosenberg, 2004).

Mice transgenic for the tumor antigen provide more accurate indication of vaccine efficacy and the risk of autoimmunity. There are several transgenic mouse models for spontaneous

adenocarcinoma (Gendler and Mukherjee, 2001), however most of these mice develop breast and prostate carcinoma and only a few gastrointestinal cancer. Most of these mouse models express well characterized human tumor antigens, such as the human epidermal growth factor receptor HER-2/neu (HER2 or c-erb-B2), mucin (MUC)-1, the carcinoembryonic antigen (CEA) or the adenomatous polyposis coli (Apc) gene, that are known to be overexpressed in a variety of human cancers as ovarian and breast cancers, pancreatic adenocarcinomas and colorectal cancers.

The RIP-Tag mouse model, which expresses the large T-antigen (Hanahan, 1985) of simian virus 40 (SV-40) under the control of the rat insulin promotor in the beta-cells of the endocrine pancreas, represents a model of spontaneous multistep tumorgenesis for pancreatic islet carcinoma. Several studies using RIP-Tag mice demonstrated that the induction of Tag-specific lymphocytes and costimulation are not sufficient for tumor rejection (Adams et al., 1987; Ganss and Hanahan, 1998; Onrust et al., 1996). However, irradiation as an inflammatory stimulus rendered solid tumors accessible for infiltration by adoptively transferred effector cells and resulted in tumor eradication (Ganss et al., 2002).

Based on the work of Leder and Muller (Muller et al., 1988), there are several models of transgenic mice that overexpress the rat (r) epithelial growth factor receptor Her-2/neu or its transformed mutated form under the control of the mouse mammary tumor virus (MMTV) promoter at a distinct period of their life and therefore develop mammary carcinoma at different ages. Vaccination-induced antibodies appear to block carcinogenesis by inhibiting receptor function and down-regulating its membrane expression in preneoplastic cells, but in most of the studied mouse lines, vaccination fails to elicit a strong, effective CTL response, probably due to the difficulty of fully breaking tolerance (Reilly et al., 2001; Foy et al., 2001; Nanni et al., 2001; Rovero et al., 2000; De Giovanni et al., 2004).

Vaccination studies on transgenic mice expressing human MUC1 under its own promotor revealed that tolerance to vaccination against MUC1 could only be broken by increasing the immunogenicity of vaccine preparation (Rowse et al., 1998; Acres et al., 2000; Koido et al., 2000; Soares et al., 2001; Carr-Brendel et al., 2000). The so called double transgenic MET mouse that is transgenic for MUC1 and in addition expresses SV-40 Tag under the control of the rat elastase promoter spontaneously develops MUC1-expressing acinar carcinoma of the pancreas. Concurrent with tumor development these mice spontaneously develop MUC1-

specific CTLs that do not stop the growth of the pancreatic tumors (Mukherjee et al., 2000). In a MUC1 transgenic mouse model for spontaneous mammary carcinoma MTT mice express the polyoma virus middle T (PyMT) oncogene under the control of the MMTV promotor and MUC1 in a tissue-specific fashion. In contrast to MET mice, no MUC1-specific CTLs can be detected in naive MTT mice, but vaccination of these mice with DC/tumor fusion cells doubles the latency period of mammary carcinoma (Xia et al., 2003).

Studies using CEA transgenic mice (Eades-Perner et al., 1994) have shown that vaccines can break tolerance to CEA and protect mice against subsequent tumor challenge by tumor cells expressing CEA without inducing any autoimmune reactions against CEA expressing tissues (Kass et al., 1999; Xiang et al., 2001; Zhou et al., 2004). Crossbreeding these mice to APC^{min} (mouse homologue of the human APC gene) mice (Moser et al., 1990; Su et al., 1992) results in the development of numerous intestinal neoplasms in adult mice with strong CEA expression in all tumor cells, as well as CEA expression in normal gastrointestinal tissue (Thompson et al., 1997; Wilkinson et al., 2001).
Only a very intense immunotherapeutic protocol induced anti-CEA host immune response that significantly suppresses intestinal tumor load and improved long-term survival of CEA.Tg/MIN mice (Greiner et al., 2002). In a recently published study, vaccination with DCs fused to tumor cells resulted in a reduced tumor growth in APC gene mutant mouse models (Iinuma et al., 2004).

1.5 Pancreatic Cancer

Pancreatic cancer is one of the most lethal human cancers and continues to be a major unsolved health problem at the start of the 21st century. Pancreatic cancer represents the fourth leading cause of cancer death in both men and woman; this number has been quite steady over the past 3-5 years (Jemal et al., 2003). Over 90% of pancratic cancers are adenocarcinomas arising from pancreatic ductal cells (Cubilla and Fitzgerald, 1976; Hruban et al., 2001; Meszoely et al., 2001); anatomy of the pancreas shown in Figure 1.2 (Bardeesy and DePinho, 2002). The overall 5-year survival rate is only 3-5%, and with a median survival of less than 6 months, a diagnosis of pancreatic adenocarcinoma carries one of the most dismal prognoses in all of medicine. Due to the lack of specific symptoms and limitations of diagnostic methods,

the disease often eludes detection during its formative stages. Whipple and colleagues reported the first “pancreaticoduodenectomy” in 1935 and surgery has since offered the only possibility of cure, although surgical intervention alone rarely achieves a curative end point (Warshaw and Fernandez-del Castillo, 1992; Baumel et al., 1994). For the 15-20% of patients who undergo potential curative resection, the 5-year survival is only 20% (Ahrendt and Pitt, 2002; Yeo et al., 1995). Some improvements in surgical outcome occur in patients who receive adjuvant treatment like chemotherapy and/or radiotherapy, although the impact on long term survival has been minimal (summarized in Bardeesy and DePinho, 2002; Li et al., 2004).

Like many other malignant diseases, pancreatic ductal carcinoma results from the accumulation of acquired mutations. In pancreatic ductal cancers oncogene (e.g. K-ras) activation, tumor suppressor gene (e.g. Trp53, p16) inactivation, and growth factor (e.g. TGF-α) and receptor (e.g EGFR, HER-2/neu) overexpression have been reported (Digiuseppe et al., 1994b; Digiuseppe et al., 1994a; Day et al., 1996; Sohn and Yeo, 2000; Sakorafas et al., 2000). A progression model has been developed that describes pancreatic ductal carcinogenesis: the pancreatic ductal epithelium progresses from normal to increasing grades of pancreatic intraepithelial neoplasia, to invasive cancer. By definition, the different stages of tumor development are termed pancreatic intraepithelial neoplasia (PanINs) and diagnostic criteria for each grade were established, summarizing histological and genetic changes (Fig. 1.3, (Hruban et al., 2000; Hruban et al., 2001)). The prevalence of these genetic alterations increases as the degree of cytological and architectural atypia in the duct lesions increases (Wilentz et al., 1998; Wilentz et al., 2000; Moskaluk et al., 1997; Goggins et al., 2000).

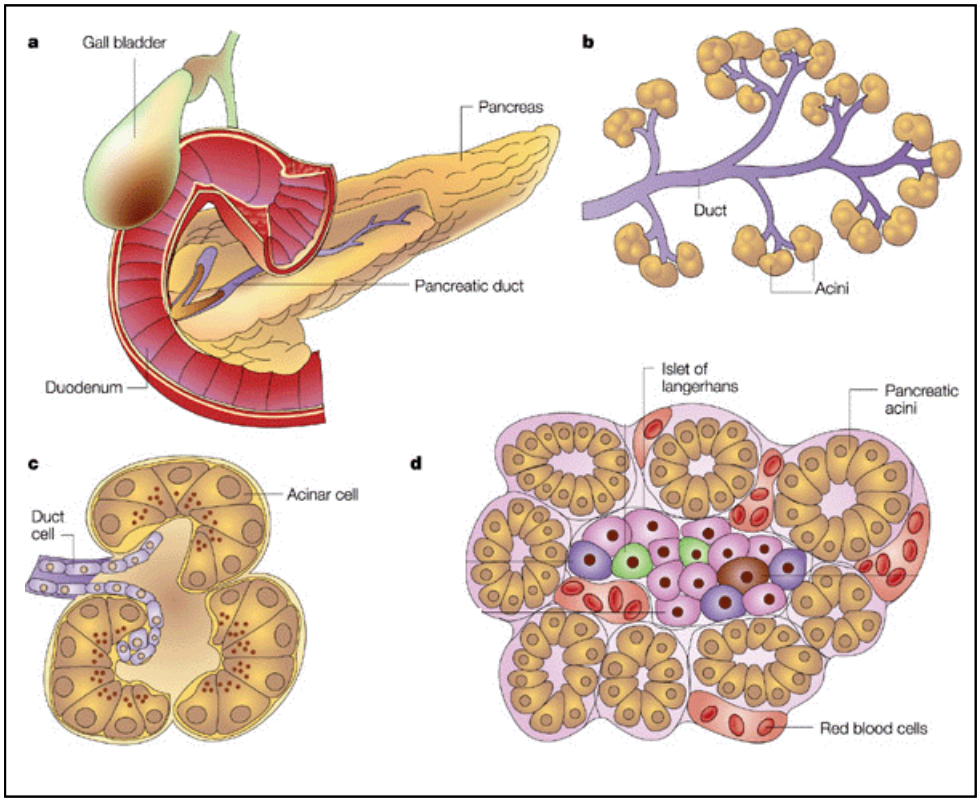

Figure 1.2: Anatomy of the pancreas. Two major physiological processes are regulated by two separate functional units of the pancreas: digestion and glucose metabolism (Githens 1993). The exocrine pancreas consists of acinar and duct cells (b and c). The acinar cells that are organized in grape-like clusters, which are the smallest termini of the branching duct system, produce digestive enzymes and constitute the bulk of pancreatic tissue. The ducts, which add mucous and bicarbonate to the enzyme mixture, form a network and empty into the duodenum (a). The endocrine pancreas that consists of four different cell types (shown in different colors) organized into the Langerhans Islet and embedded within acinar tissue (d), sectretes hormones (insulin and glucagon) into the bloodstream (adapted from Bardeesy 2002).

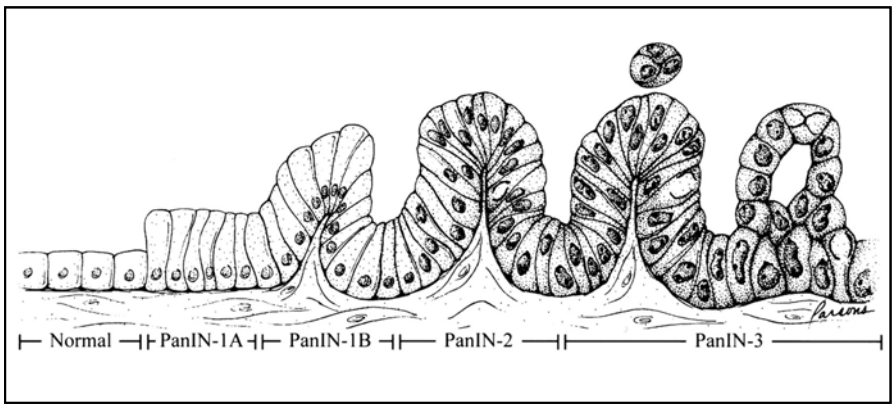

Fig 1.3. Progression model for pancreatic cancer. The pancreatic ductal epithelium progresses from normal to increasing grades of pancreatic epithelial neoplasia, to invasive cancer through a series of histological defined precursos (PanINs = pancreatic intraepithelial neoplasia) (adapted from Hruban 2000).

1.5.1 Immunotherapy for pancreatic cancer

Despite efforts in the past 50 years, conventional treatment approaches, such as surgery, radiation, chemotherapy, or combinations of these, have had little impact on the course of adenocarcinoma of the exocrine pancreas and five-year survival rates remain below 5 % (Li et al., 2004). One promising alternative therapy is immunotherapy (Greten and Jaffee, 1999) that can be integrated with surgery, radiation therapy and chemotherapy. Recently, an allogeneic granulocyte macrophage colony-stimulating factor-secreting tumor vaccine was tested in patients who underwent a Whipple procedure. The vaccine was demonstrated to be safe and to induce tumor-specific immunity (Jaffee et al., 2001). Immunization with heat shock proteins (HSP) isolated from pancreatic tumor cells is another important approach that is undergoing clinical testing (Janetzki et al., 2000). Antibody approaches targeting pancreatic cancer-associated tumor antigens such as CEA, MUC-1, and mutated KRAS have also undergone clinical testing. These antibodies have been employed in two ways for treatment: The first is treatment with antibodies conjugated to immunotoxins or radionucleotids that are upon binding of the antibody directly delivered to the cancer cell. Second, treatment with antibodies alone can result in the antibody acting as growth factor inhibitor, thereby

decreasing cellular signaling after receptor engagement. Antibodies alone as treatment can also induce antibody dependent cell cytotoxicity via activation of the complement pathway. (Nishihara et al., 2000; Bruns et al., 2000; Nielsen et al., 2000; Staib et al., 2001; Fan and Mendelsohn, 1998). Active immunization with peptide- and protein-based vaccines for MUC1, KRAS as well as immunization with recombinant virus encoding the antigens such as CEA and with loaded or transfected dendritc cells are also clinical trials that are in progress (Gjertsen et al., 2001; Gjertsen et al., 1995; Goydos et al., 1996; Marshall et al., 2000; Pecher et al., 2002). (summarized in Laheru et al., 2001, Kawakami et al., 2004). In a recently published study, a pancreatic tumor-associated antigen mesothelin was identified as a relevant target for vaccine-induced T cell responses. In this approach, the correlation of *in vitro* T cell responses with immunological responses in vaccinated pancreatic cancer patients was validated and it was suggested that cross-presentation of mesothelin may have clinical relevance (Thomas et al., 2004). However until today immunotherapeutic approaches have not been tested in a murine model of pancreatic adenocarcinoma due to the lack of an appropriate model.

1.5.2 Transforming growth factor -α

Transforming growth factor alpha (TGF-α) is a member of the epidermal growth factor (EGF) family of proteins. It shares structural and functional characteristics with EGF and is a ligand of the EGF receptor (EGFR), a protein tyrosine kinase that is involved in the mitogenic signal transduction pathway of cells. TGF-α is synthesized as a 160 amino acid glycosilated and palmitoylated transmembrane precursor, termed proTGF-α (Lee et al., 1985; Blasband et al., 1990). The mature 50 amino acid TGF-α is released from the extracellular domain by proteolytic cleavage. Larger soluble forms of TGF-α representing incomplete processed intermediates have also been identified. This heterogeneity appears to be due to both the type of degree of ectoglycosylation and the preference for different sites of proteolytic cleavage of the precursor. Mutant forms of the integral membrane pro-TGF-α, which cannot be processed to mature peptide, can still initiate signal transduction through EGFR on the surface of contiguous cells *in vitro* (Wong et al., 1989; Bringman et al., 1987). Elevated expression of TGF-α has been found in neoplastic tissues or cell lines derived from spontaneous human

tumors or induced animal tumors, suggesting that TGF-α contributes to neoplastic growth through autocrine and paracrine mechanisms (Maruvada and Levine, 1999; Derynck, 1988).

In fact, increased expression of the precursor for TGF-α in transgenic mice causes hyperplasia of several tissues and even neoplastic transformation (Jhappan et al., 1990; Matsui et al., 1990; Sandgren et al., 1990).

1.5.3 The tumor suppressor gene Trp53

The wild type p53 gene is a tumor suppressor gene which encodes a protein that regulates a cell cycle checkpoint and the induction of programmed cell death (apoptosis). In normal cells, the p53 protein levels are low. DNA damage, cell stress or the aberrant expression of some oncogenes trigger the increase of the p53 protein (Yonish-Rouach et al., 1991). As a potent transcription factor p53 serves as the "guardian of the genome" (Lane, 1992). Expression of high levels of wild-type p53 has three major outcomes: growth arrest, DNA repair and apoptosis. Growth arrest stops the progression of the cell cycle, preventing replication of damaged DNA. During this growth arrest, p53 may activate the transcription of proteins involved in DNA repair. Apoptosis is the "last resort" to avoid proliferation of cells containing abnormal DNA (Marx, 1994; Ko and Prives, 1996). The cellular concentrations of p53 must be tightly regulated. In cells, p53 can associate with MDM2, which is the major regulator of p53. Binding to MDM2 keeps p53 levels low and holds apoptosis in check (Alarcon-Vargas and Ronai, 2002). Mutations of the p53 gene disable this "emergency brake" on cell proliferation and lead to genetic instability. Missense mutations in the p53 gene (generally alterations of the DNA-binding domain), which inactivate its growth suppressing activities, have been observed in more than 50% of pancreatic adenocarcinomas (Rozenblum et al., 1997) and in over 60% of all human tumors (Hollstein et al., 1991; Chang et al., 1995).

Mice that are homozygous null for the Trp53 gene, although developmentally competent, are highly predisposed to cancer and develop tumors in multiple tissues (Jacks et al., 1994; Donehower et al., 1992). The loss of p53 does not alter lymphopoiesis or significantly impairs immune responses (Donehower et al., 1992; Wen et al., 2001; Grayson et al., 2001; Bachelier et al., 2003).

1.5.4 Mouse models for exocrine pancreatic cancer

Spontaneously or following carcinogen administration, pancreatic cancer is rarely observed in mice. Genetic engineering has allowed the generation of mouse strains that harbour germline oncogenic lesions that are found in human adenocarcinomas. Most of these mouse models of exocrine pancreatic tumor development do not reproduce the ductal phenotype of human pancreatic adenocarcinoma but display predominantly acinar characteristics, mixed acinar-ductal tumors, or cystic tumors (Quaife et al., 1987; Ornitz et al., 1987; Sandgren et al., 1991; Glasner et al., 1992; Mukherjee et al., 2000; Tevethia et al., 1997; Bardeesy et al., 2002; Grippo et al., 2003). In a new a transgenic mouse model activated mutant Kras and tumor suppressor gene Ink4a/Arf deficiency cooperate to produce metastatic pancreatic ductal adenocarcinoma with striking resemblance to the human disease (Aguirre et al., 2003).

1.5.4.1 TGF-α $p53^{-/-}$ mice

Transgenic mice overexpressing transforming growth factor alpha (TGF-α) under the control of the rat elastase promotor (EL-TGF-a-hGH, Fig. 1.4) show a transdifferentiation of acinar cells to duct-like cells, which represent premalignant lesions (Sandgren et al., 1990; Wagner et al., 1998), accompanied by an induction of the EGFR expression in the resulting metaplastic ducts that proliferate due to an autocrine loop. About 30% of TGF-α transgenic mice develop malignant pancreatic tumors with a mean tumor-free survival of 410 days, similar to the human disease regarding cellular differentiation, growth characteristics, and genetic alterations. Crossbreeding TGF-α transgenic mice to p53 deficient mice results in increased incidence of pancreatic tumors (Fig. 1.5, (Wagner et al., 2001)). Heterozygous loss of p53 resulted in an increased incidence of pancreatic tumors (77%) and a decreased tumor-free survival to 220 days in TGF-α $p53^{+/-}$ animals. 100% of TGF-α $p53^{-/-}$ mice developed pancreatic tumors within 120 days after birth. Some pancreatic tumors form ductal structures in dense connective tissues, others show frequent mitotic figures surrounded by sparse fibrosis. Malignant ascites, local invasive growth with duodenal obstruction, and metastasis to the liver and lung is also observed in TGF-α transgenic mice crossbred to p53-null mice (Wagner et al., 2001). The tumor spectrum of p53 deficient mice is dominated by lymphomas and sarcomas (Jacks et al., 1994). This spectrum shifted to ductal pancreatic cancer in

crossbred animals. Only 3 of 90 mice developed pancreatic tumors and sarcomas. The pancreatic tumors bear similar genetic changes, as were observed in human pancreatic cancer, including loss of the wild-type allele of p53 and inactivation of the putative tumor suppressor $p16^{ink4A}$ (Serrano et al., 1996) in TGF-a $Trp53^{+/-}$ mice (Wagner et al., 2001). Thus, TGF-α p53 deficient mice represent a murine pancreatic cancer model that recapitulates several pathomorphological features and genetic alterations of the human disease.

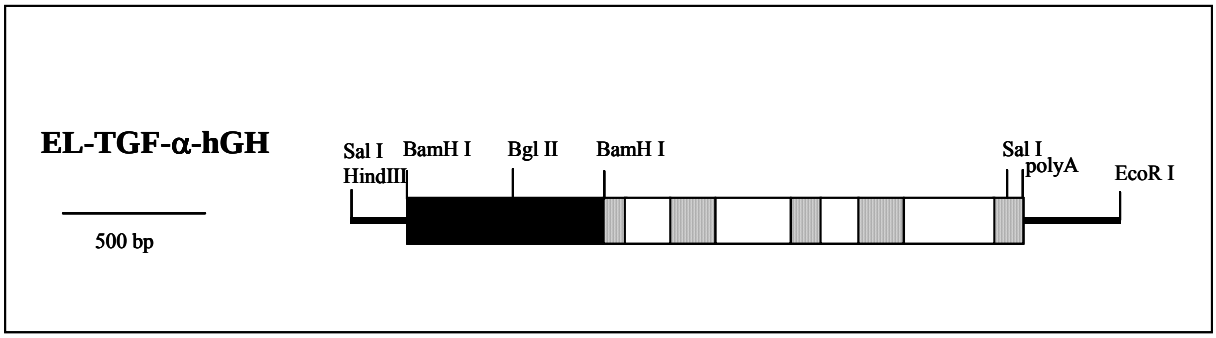

Figure 1.4. EL-TGF-α-hGH fusion gene contruct for the generation TGF-α transgenic mice. Solid rectangle represents TGF-α cDNA sequences, stippling represents hGH exon sequences, and open rectangles represent introns (adapted from Sandgren 1990).

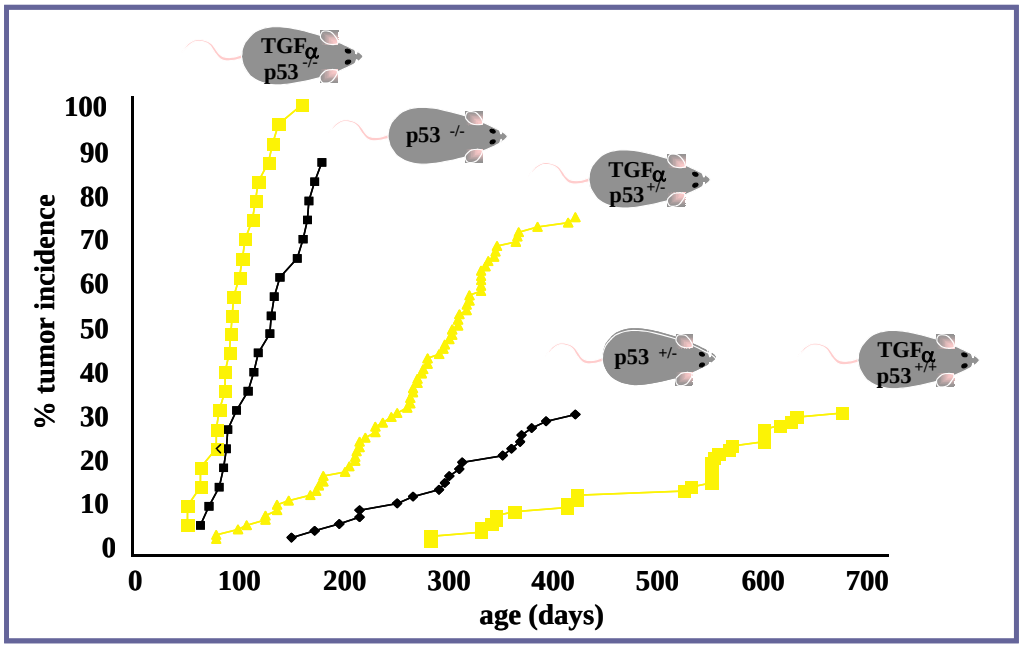

Figure 1.5: Cumulative incidence of tumors in TGF-α, $p53^{+/-}$, TGF-α $p53^{+/-}$, $p53^{-/-}$ and TGF-α $p53^{-/-}$ mice (adapted from Wagner 2001)

1.6 Aims of the study

TGF-α Trp53$^{-/-}$ mice have been decribed as the first murine model for the development of ductal adenocarcinoma of the pancreas with pathomorphological features and genetic alterations similar to the human disease (Wagner et al., 2001). To perform immunological studies on pancreatic adenocarcinoma in a murine mouse model, TGF-α Trp53$^{-/-}$ mice were bred on C57Bl/6 background. In order to see if these mice are a suitable model for the development of immunotherapy approaches, extensive morphological and immunological analysis of TGF-α Trp53$^{-/-}$ mice should be performed. The aims of this study were:

- Monitoring of the development of spontaneous pancreatic tumors in TGF-α Trp53$^{-/-}$ mice on C57Bl/6 background. Morphological and immunohistological examination of the pancreatic tumors to define the degree of tumor development and to analyze tumors for infiltrating lymphocytes.

- Generation and characterization of cell lines derived from pancreatic adenocarcinoma of TGF-α Trp53$^{-/-}$ mice. Comparison of *in vivo* growth kinetics of these cell lines in immune competent and in immune deficient mice.

- Vaccination studies with pancreatic cell lines. Definition of the role of TGF-α as potential tumor antigen.

- Analysis of tumor specific cellular and humoral immune responses in TGF-α Trp53$^{-/-}$ mice with premalignant and malignant lesions.

- Investigation of possible tumor escape mechanisms of solid tumors.

- Finally, suitable immune therapy approaches against pancreatic adenocarcinoma in TGF-α Trp53$^{-/-}$ mice should be evaluated.

2 MATERIALS AND METHODS

2.1 Mice

The p53-deficient mice and the transforming growth factor-α (TGF-α) transgenic mice that express a TGF-α-human growth hormone (hGH) fusion gene under the control of the elastase (EL) promotor (EL-TGF-α-hGH transgenic mice, line no. 2261-3) have been previously described (Jacks et al., 1994; Sandgren et al., 1990). TGF-α transgenic mice were kindly provided by Dr. Roland Schmid, TU München. TGF-$\alpha^{+/-}$Trp53$^{-/-}$ mice were obtained by crossing TGF-α transgenic mice with Trp53$^{-/-}$ mice. IFN-γ-deficient mice (B6.129S7-Ifnγ^{tm1Ts}) were kindly provided by Dr. Siegfried Weiss, GBF, Braunschweig. TGF-α transgenic, p53-deficient, IFN-γ-deficient and nu/nu mice were bred in the animal facilities of the Medizinische Hochschule Hannover. Mice were backcrossed for a minimum of 10 generations on C57Bl/6 background.
RAG1$^{-/-}$ mice were kindly provided by Dr. Jan Buer, GBF, Braunschweig. CD1$^{-/-}$ mice were kindly provided by Dr. Stefan Kaufmann, MPI for Infection Biology, Berlin. C57Bl/6 mice were obtained from Charles River (Sulzfeld, Germany). SCID.beige mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA). All mice were kept under specific pathogen-free conditions, and all experiments were conducted according to the German animal protection law.

2.1.1 PCR screening

Genotype of mice used in this study was determined by genomic PCR of tail biopsies. PCRs were performed using Hot Star Taq (Qiagen, Hilden, Deutschland) and were initiated at 95°C for 15 min. The status of p53 was assessed by a multiplex PCR using the following primers: 5`p53 (5`-CCCGAGTATCTGGAAGACAG-3`) and 3`p53 (5`-ATAGGTCGGCGGTTCAT-3`), 5`neo (5`-CTTGGGTGGAGAGGCTATTC-3`) and 3`neo (5`-AAGTGAGATGAC AGGAGATC-3`). A 600 bp fragment of wt p53 and a 280 bp fragment of neomycin were amplified by PCR as follows: 94°C for 30 s, 58°C for 1 min, 72°C for 1 min. PCR was carried out for 30 cycles. The presence of TGF-α transgene was determined using hGH gene specific primers: 5`hGH (5`-GGCTTTTTGACAACGCTATG-3`) and 3`hGH (5`-TAGGAGGTC ATAGACGTTGC-3`) (600 bp). The PCR was performed for 40 cycles according to the following program: 94°C for 30 s, 58°C for 1 min, 72°C for 1 min. IFN-γ $^{-/-}$ mice were

identified using multiplex PCR with 3 different primers: G-IFN725 (5`-TCAGCGCAGGGG CGCCCGGTTCTTTT-3`) and G-IFN482 (5`-AGAAGTAAGTGGAAGGGCCCAGAAG-3`) for wt IFN-γ (260 bp) and G-IFN484 (5`-AGGGAAACTGGGAGAGGAGAAATAT-3`) and G-IFN484 for neo-disrupted IFN-γ (1 Kb). Conditions were: 94°C for 30 s, 58°C for 1 min and 72°C for 1 min; 35 cycles.

2.2 Tumor cell lines

Tumor cell lines were generated from pancreatic tumors of 6 TGF-α/p53$^{-/-}$ mice by fine needle aspiration or homogenization. Fibroblast overgrowth was controlled by differential trypsinization. Established primary cultures were passaged by trypsinization when each culture formed an 80% to 100% monolayer. Established mPAC (murine pancreatic adenocarcinoma cell lines) 1-25 derived from TGF-α/p53$^{-/-}$ mice were used for further *in vitro* and *in vivo* characterization and analysis. The main *in vivo* experiments of this study were performed with mPAC-6 and the once *in vivo* passaged (ivp) variant mPAC-2ivp.

B78H1K^{b}D^b, a derivative of B78H1, a MHC class I-negative variant of B16 melanoma of a C57Bl/6 mouse (Graf, Jr. et al., 1984), was transduced with the K^{b}D^b gene (Levitsky et al., 1994). Hepa1-6 is a murine hepatom derived from C57L/J mouse (Darlington et al., 1986). RMA is a mouse T cell lymphoma of C57Bl/6 origin (Karre et al., 1986). NIH3T3 is a murine fibroblast cell line. All cell lines were grown in DMEM complete medium (10% FCS, 100 U/ml Penicillin, 100 µg/ml Streptomycin, 2 mM L-Glutamin, 1% NEEA and 1 mM Sodiumpyruvat under standard culture conditions (37°C, 5% CO_2). mPAC were tested for mycoplasma contamination using nested PCR. Two consecutive PCR runs were performed to increase the sensitivity of the PCR, employing a nested set of general, mycoplasma specific primers (Wirth et al., 1994; Hopert et al., 1993). As few as 1-2 mycoplasma genome copies are detectable using this nested PCR. Some cells were subjected to 2-weeks treatment with 40 µg/ml ciprofloxacin (Ciprobay 200; Bayer, Leverkusen, Germany) (Uphoff et al., 2002).

2.2.1 Cloning of cell lines

Established mPAC were plated in 96-well-plates under limiting dilution conditions and only wells bearing single colonies were expanded for further experiments.

2.3 Transfection and Retroviral Transduction

To construct pSecTagTGF-α, rat cDNA of TGF-α was cloned out of TGF-α pGEM-4Z (kindly provided by David Lee) into pSecTag2A/Hygro vector (Invitrogen, Karlsruhe, Germany). Cells used as target cells in cytotoxicity assays (B78H1kbDb and Hepa1-6) were transiently transfected with pSecTagTGF-α using Lipofectamine (Invitrogen). TGF-α expression of the transfected cells was verified by Western Blot using anti-TGF-α or anti-*myc* antibody for detection (see below for detailed protocol). For retroviral transduction, supernatant of GM-CSF-retrovirus (CMMP GM-CSF/IRES/GFP) producing cells (kindly provided by Dr. Christoph Klein, MHH) was applied at different ratios to mPAC in the presence of 8 μg/ml Polybrene . After overnight (o.n.) incubation, the infection supernatant was discarded and fresh medium was added to the cells. After another 48-72 hours of culture, cells were analyzed by fluorescence microscopy and FACS analysis for Green Fluorescent Protein (GFP) expression. For detection of GM-CSF expression, mPAC were seeded at a defined density in a defined volume of cell culture medium and the supernatant of the transduced mPAC was analysed after o.n. culture for GM-CSF by ELISA (Quantikine kit, R&D, Wiesbaden, Germany) according to manufacturer`s recommendations.

2.4 Western Blot

To detect protein expression, pancreata, mPAC, control cell lines or cell lines expressing recombinant proteins were homogenized in ice cold lysis buffer (150 mM NaCl, 50 mM Tris-HCl pH 7,2, 2 mM EDTA and 1% NP40). The buffer was supplemented with a protease inhibitor cocktail (Complete Mini, Roche Diagnostics, Mannheim, Germany). Protein concentrations were measured using the Bradford method (Bradford reagent, BioRad, München, Germany). Equal amounts of total protein were separated on 10-15% SDS gels and

analysed by Western Blot for Cytokeratin 8/18 or TGF-α expression using a polyclonal guinea pig anti-Cytokeratin 8/18 (GP11, Progen, Heidelberg, Germany) or monoclonal anti-TGF-α antibodies (134A-2B3, Oncogene, Cambridge, MA, USA or MF9, Neomarkers, Fiemont, CA, USA). Expression of recombinant proteins containing the *myc* epitope was detected in Western Blot using an Anti-*myc*-HRP antibody (Invitrogen). Membranes were blocked in 5% nonfat, dry milk and 0.1% Tween-20 in phosphate-buffered saline (PBS) for one hour at room temperature or o.n. at 4°C, incubated with the primary antibody (α-CK8/18 1:2000 and α-TGF-α 1:20) in blocking solution for one hour at room temperature or overnight at 4°C, washed three times for 10 minutes in PBS-Tween-20, and incubated with a 1:10000 dilution of a horseradish peroxidase-labeled secondary antibody (anti-guinea pig IgG (Sigma, Seelze, Germany)) or anti-mouse IgG (H+L) (Promega, Mannheim, Germany) in 10% blocking solution for 1 hour. After three 10-minute washes in PBS-T, binding of antibody was visualized with enhanced chemiluminescence reagent (Amersham, Braunschweig, Germany).

2.5 Preparation of RNA and reverse transcription (RT PCR)

Total cellular RNA was isolated from 1 x 10^7 mPAC or control cell lines using RNeasy Kit (Qiagen, Hilden, Germany) according to standard protocols. Residual chromosomal DNA was digested with DNAse I (Live technologies, Karlsruhe, Germany). First strand cDNA was synthesized from total RNA (2µg) in a 20 µl reaction using Oligo(dT) primer and the enzyme Superscript^{TM}II (Live Technologies, Karlsruhe, Germany) according to manufacturer`s recommendations. One µl of the primer was mixed with 2 µg total RNA, and sterile double distilled water was added to a total volume of 12 µl. The mixture was incubated at 70°C for 10 min and quickly chilled on ice. After a centifugation step 4µl of 5 x first strand buffer, 2 µl of 0,1 M DTT and 1 µl of 10 mM dNTP-mix were added. The contents were gently mixed and incubated at 42°C for 2 min, 1 µl Superscript^{TM}II was added. The reaction was continued for 50 min at 42°C followed by inactivation of reverse transcriptases. PCR was initiated at 95°C for 15 min (Hot Star Taq). The synthesized cDNA was analyzed using primers specific for Cytokeratin 18: 5`CK18 (5`-TGGTACTCTCTTCAATCTGCTG-3`) and 3`CK18 (5`-CTCTGGATTGACTGTGGAAGTG-3`) and Cytokeratin 19: 5`CK19 (5`-CATGGTTCT TCTTCAGGTAGGC-3`) and 3`CK19 (5`-GCTGCAGATGACTTCAGAACC`-3) (174 bp). The PCR was performed for 30 cycles according to the following program: 94°C for 15 s,

54°C for 30 s, 72°C for 30 s. A 158 bp fragment of TGF-α was amplified using the following primers: 5`TGF-α (5`-GTGGTGTCTCACTTCAACAAG-3`) and 3`TGF-α (5`-TGCCAG GAGATCTGCATGCTC-3`). Conditions were: 94°C for 30 s, 60°C for 1 min, 72°C for 1 min; 30 cycles, and for human Growth Hormone: 5`hGH (5`-CCGACACCC TCCAACAGGGA-3`) and 3`hGH (5`-CCTTGTCCATGTCCTTCCTG-3`) (Raccurt et al., 2002) the PCR conditions were: 94°C for 30 s, 58°C for 1 min, 72°C for 1 min; 35-40 cycles.

2.6 Tumor transplantation

To analyse the *in vivo* growth of the generated cell lines derived from TGF-α/Trp53$^{-/-}$ mice, 1 x 10^7 mPAC were injected subcutaneously (s.c.) in 200 µl sterile PBS in the hind flank of mice. For re-challenge experiments, 1-10 x 10^6 mPAC were injected in the opposite flank.
Tumor development was monitored every other day by measuring the tumor with the metric caliper. For ethical reasons, animals were sacrificed when tumors reached a diameter greater than 15 mm.

2.7 Preparation of single cell suspensions

Single cell suspensions were obtained by flushing spleens with DMEM complete medium followed by red blood cell lysis using erythrocyte lysis buffer (Qiagen) or by disaggregation of mesenteric and inguinal lymph nodes by using the back of a syringe`s plunger. Cells were passed through a mesh with a 100 µm pore size and were washed with DMEM complete medium. Blood obtained from the tail artery or by cardiac puncture was collected in PBS containing Heparin (50 U/ml) followed by separation on ficoll gradients. Single cell suspension of tumors were prepared by mechanical dissociation and collagenase/dispase treatment for 45-60 min at 37°C (200 U/ml collagenase (Sigma) /0,7 U/ml dispase (Roche Diagnostics)). Cell-cell interactions were disrupted by adding EDTA (0,1 M) during the last 5 min of incubation, cells were washed and passed through a mesh.

2.8 Antibodies and flow cytometry

The monoclonal antibodies (mAb) against H-2Kb (AF6-88.5), I-A/I-E (M5/114.15.2), CD3 (145-2C11), CD4 (L3T4) (GK1.5 and RM4-4), CD8a (Ly-2) (53-6.7), CD11b (M1/70), CD11c (HL3), CD19 (1D3), CD25 (7D4), CD45R/B220 (RA3-6B2), CD49b/Pan-NK Cells (DX5), NK1.1 (PK136), used as biotin, fluorescein (FITC), phycoerythrin (PE), allophycocyanin (APC) or peridinin chlorophyll protein (PerCP) conjugates, were obtained from BD PharMingen (Heidelberg, Germany). Biotinylated antibodies were revealed by PE-streptavidin- or APC-streptavidin- conjugates (BD PharMingen). Flow cytometry was carried out using a FACSCalibur and CellQuest software (Becton Dickinson).
For *in vivo* depletions, antibodies were produced in serum free medium from hybridoma culture supernatants using the Integra^{TM} cell line CL1000 system (Integra Bioscience, Chur, Switzerland), precipitated with ammonium sulfate and dialyzed against PBS.

2.9 Depletions of cell subsets *in vivo*

Mice were injected intraperitoneally (i.p.) with either 100 µg of purified rat monoclonal antibody GK1.5 (anti-CD4), 2.43 (anti-CD8), or 1 mg PC61 (anti-CD25) with a one-day interval. Injection of monoclonal antibodies was started at day 3 prior to the injection of tumor cells and then continued every other day. Depletion of the different subsets was confirmed by FACS-analysis by using the RM4-4 (CD4), 53-6.7 (CD8), and 7D4 (CD25) antibodies (BD PharMingen), which do not compete with the depleting antibodies for binding to the different cell subsets. Depletion was maintained by continuing the antibody injections twice weekly for the duration of the tumor challenge experiments.

2.10 Serology

To determine mPAC-specific serum immunoglobulin (Ig)G titers, mPAC-6, mPAC-2ivp and irrelevant tumor cell lines were stained using serum (1:50 dilution) taken of mPAC bearing mice as primary antibody. A FITC conjugated goat anti-mouse pan IgG secondary antibody (Southern Biotec, Birmingham, AL, USA) was used to detect bound serum IgG.

2.11 Assessment of MHC I expression on tumors

To evaluate the status of MHC I expression on mPAC grown *in vivo* after s.c. injection, single cell suspension of tumors were prepared by mechanical dissociation and collagenase/dispase treatment (see above for detailed protocol). Cells were washed, passed through a mesh, stained for MHC I and were analyzed by FACS.

2.11.1 Treatment of cells with Interferon-γ

For analysis of induction of MHC class I expression in tumors, recombinant murine IFN-γ (Peprotech, London, UK) was added to tissue culture medium at a final concentration of 100 U/ml during 24 h of culture. Induced MHC class I expression was then revealed by FACS analysis.

2.12 Cytotoxicity Assay

mPAC-specific cytotoxicity was assayed in a standard chromium release assay. In brief, mice were immunized by s.c. injection of irradiated (50 Gy) mPAC or irrelevant tumor cell line. After 14 days pooled mesenteric and inguinal lymph nodes and spleen cells were restimulated for 5 days at 4 x 10^6/well in a 24-well culture dish with 1 x 10^5 mitomycin-treated mPAC (1 mg mitomycin/1 x 10^7 cells in 10 ml for 1,5 hours) in RPMI complete medium containing 50 µM β-mercaptoethanol. 10 U/ml IL-2 was added at day 2. After 5 days the restimulated cells were harvested, washed and incubated at different ratios with radioactively labeled (100 µCi ^{51}Cr/1 x 10^6 cells) mPAC or irrelevant tumor cells at 5000/well in 96-well-v-bottom culture dishes for 4 h. 50 µl supernatant from each well mixed with 150 µl Optiphase Super-Mix (Wallac, Turku, Finland) was counted in the gamma counter (Wallac). Specific lysis was calculated as follows: (experimental cpm – spontaneous cpm)/(maximum cpm – spontaneous cpm) x 100.

2.13 IFN-γ capture assay

To detect mPAC-specific IFN-γ secretion, the Cytokine secretion assay for murine cells (Miltenyi Biotec, Bergisch Gladbach, Germany) was used according to manufacturer`s recommendations. Briefly, 5 x 10^6 pooled mesenteric and inguinal lymph nodes and spleen cells of TGF-α/p53 deficient mice or of mice immunized with irradiated mPAC or an irrelevant cell line were stimulated over night (max 16 h) with 1 x 10^5 mitomycin treated mPAC in 48 well culture dishes. After washing, an IFN- γ-specific catch reagent was attached to the cell surface of all leukocytes. The cells were labeled for 5 min on ice, then incubated for 45 min at 37°C, turning the tube every 5 min to allow cytokine secretion. The secreted IFN- γ binds to the IFN- γ catch reagent on the positive, secreting cells. Cross-staining was avoided by keeping the cell density at 1 x 10^5 cells/ml. IFN- γ secreting cells were subsequently labeled with a second IFN- γ-specific antibody, the IFN- γ detection antibody conjugated to PE. The cells were counterstained with monoclonal antibody against CD8 (FITC labelled CD8a, Ly-2, 53-6.7) and the frequency of IFN- γ secreting $CD8^+$ T cells was determined by FACS analysis.

2.14 Intracellular Cytokine staining

Intracellular staining for IFN- γ was performed using the Cytofix/Cytoperm kit (Becton Dickinson) according to manufacturer`s instructions. Briefly, pooled mesenteric and inguinal lymph nodes and spleen cells were restimulated as described for IFN- γ capture assay and in the last 4h of restimulation GolgiPlug (BrefeldinA) was added to the culture. Cells were first stained for surface expression of CD8 (FITC labelled CD8a, Ly-2, 53-6.7) and then fixed and permeabelized with Cytofix/Cytoperm buffers. Intracellular IFN- γ was detected using an anti- IFN- γ–PE antibody (XMG1.2, BD PharMingen). The frequency of IFN- γ positive CD8 T cells was determined by FACS analysis.

2.15 Cytokine ELISA

To evaluate the cytokine secretion of TILs, tumors were explanted at different time points after s.c. injection. 30-100 mg tumors were reduced to small pieces using scalpel and forceps and incubated o.n. in 0,5-2 ml (depending on the size of the tumor) DMEM complete medium at 37°C, 5% CO_2 in 24-well-plates.

Cytokine secretion of TILs or of restimulated lymphocyte cultures was determined using DuoSet ELISA (R&D) specific for IFN-γ, IL-2 or IL-4, ELISA Kits from BioSource (Camarillo, CA, USA) specific for IL-10 or IL-12 and an ELISA KIT from Promega specific for TGF-β. All ELISA were performed according to manufacturer`s recommendations. Briefly, Maxisorb plates were coated over night with a cytokine-specific antibody, or precoated plates were used (BioSource). The next day, ELISA was performed following the manufacturer`s protocol. All ELISA performed in this study were sandwich ELISA using HRP-conjugates for detection. Binding of HRP conjugates was visualized with TMB solution (BioRad). The optical density was determined by using a microplate reader set to 450 nm.

2.16 Cytometric Bead Array (CBA)

Cytokine production detected in ELISA was confirmed and additional cytokines were detected by using the mouse inflammatory CBA kit (Becton Dickinson) that can simultaneously and quantitatively measure 6 different cytokines (IL-6, IL-10, IL-12, IFN-γ, monocyte chemoattractant protein-1 [MCP-1] and tumor necrosis factor-α [TNF-α]) in a single sample. Different CBA beads are stably labeled with a fluorescent dye whose emission is read at ~ 650 nm (FL 3). Each different group of beads is labeled with a discrete level of fluorescent dye so that it can be distinguished by its mean fluorescent intensity upon FACS analysis. In addition, the beads have been covalently coupled with capture antibodies specific for the different cytokines.

CBA was performed following the manufacturer`s recommendations. In brief, the CBA capture beads are mixed with PE coupled detection antibodies (specific for the definite cytokine) and standards, controls, or test samples, to form sandwich complexes. The mixture was incubated for 2 hours at room temperature. Unbound detector antibody-PE reagent was removed by a single washing step before data acquisition was performed by flow cytometry

using a FACSCalibur. Acquired data were analysed using the Becton Dickinson Cytometric Bead Array software.

2.17 Histology

Tumors were immersion fixed in buffered formalin, embedded in paraffin, sectioned at 4 μm thickness and stained with hematoxilin and eosin (H&E).

Freshly excised tumors were snap frozen in liquid nitrogen. Sections (6 μm) of frozen tissues were fixed in methanol/acetone and stained with purified anti-CD4, anti CD8 or antibodies (GK1.5, 53-6.72, BD PharMingen), followed by incubation with rabbit anti-rat IgG (DakoCytomation, Copenhagen, Denmark) and visualized using the APAAP system (DakoCytomation). Sections were counterstained with hemalaun. Mean values of tumor infiltrating lymphocytes were calculated from several vision fields on three different sections.

3 RESULTS

3.1 Development of pancreatic tumors in TGF-α p53$^{-/-}$ mice on C57Bl/6 background

For immunological studies it is essential to work with mice on a defined genetic background. Therefore, TGF- α transgenic mice on C57Bl/6 background were mated to p53 deficient mice on C57Bl/6 background. In the F2 generation 100% of TGF-α p53$^{-/-}$ mice developed malignant tumors within 124 days after birth (Fig. 3.1). Some TGF-α p53$^{-/-}$ mice (4 of 13) developed pancreatic tumors already after 68-93 days, but the majority (9 of 13) of TGF-α p53$^{-/-}$ mice did not show obvious signs of wasting disease before day 100. Heterozygous littermates did not develop tumors within this period of time.

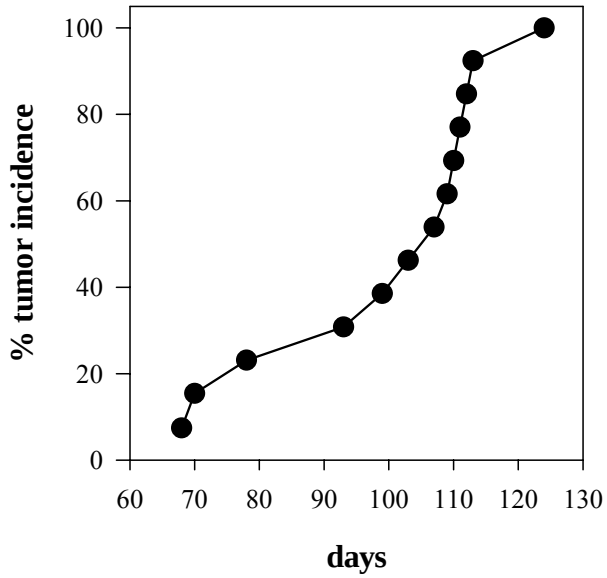

Figure 3.1. Cumulative tumor incidence in TGF-α p53$^{-/-}$ mice on C57Bl/6 background (n=13). Mice were sacrificed at depicted time points with obvious signs of wasting disease.

Histological analysis of the pancreatic tumors explanted from TGF-α p53$^{-/-}$ mice on C57Bl/6 background revealed the typical structures of ductal pancreatic adenocarcinoma with irregular cellular morphology compared to the normal pancreas of a wildtype C57Bl/6 mouse (Fig. 3.2). This includes pleomorphic nuclei with abnormal chromatin patterns and frequent mitotic

figures in ductal structures. Macroscopic examination of pancreatic tumors revealed a papillary to cystic phenotype (Fig. 3.2 C).

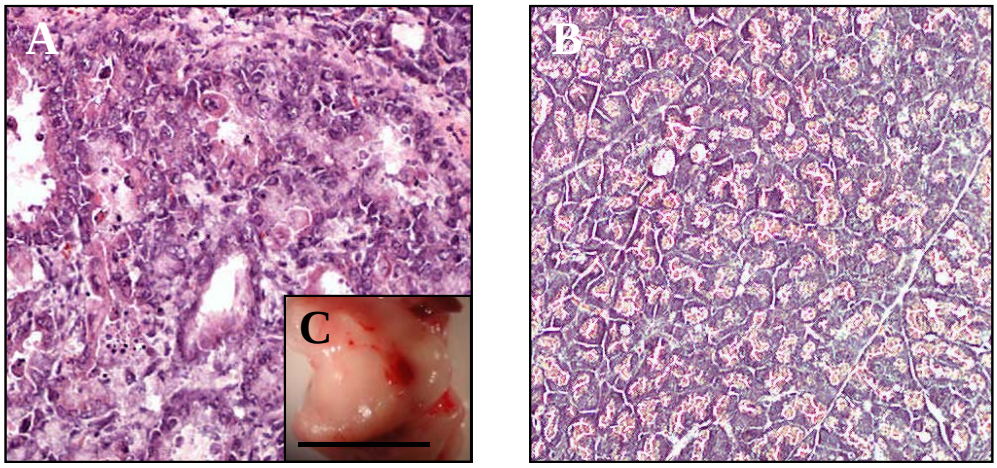

Figure 3.2. Histological analysis of a representative pancreatic tumor of a TGF-α p53$^{-/-}$ mouse on C57Bl/6 background (A) compared to a wildtype pancreas (B). Note the histological appearance of the pancreatic tumor with ductal structures and irregular cellular morphology. Tissues (8 µm) were stained with hematoxilin and eosin (original magnification 100x). (C) Macroscopic examination of a pancreatic tumor (bar, 10 mm).

3.1.1 Establishment of murine pancreatic adenocarcinoma cell lines (mPAC)

25 pancreatic tumor cell lines were generated from pancreatic tumors of 6 different TGF-α/p53$^{-/-}$ mice. Established primary cultures have been maintained in culture for more than 20 passages. To control fibroblast overgrowth during the early phase of establishing these pancreatic cell lines, the fibroblasts were repeatedly differentially trypsinized while the malignant cells stayed attached to the tissue culture flask. Established primary cultures grew in monolayers (Fig. 3.3 A). Established mPAC were characterized and analysed *in vitro* and *in vivo*.

To confirm, that the established mPAC derived from malignant epithelial tumors, mPAC were analysed for Cytokeratin 8/18 expression (Fig. 3.3 B). Cytokeratin 18 expression was confirmed by RT PCR (data not shown). Some Cytokeratin 8/18 positive mPAC were further analysed for Cytokeratin 19 expression detected by RT-PCR (174 bp), indicating a ductal

origin of mPAC (Fig. 3.3 B). Table 3.1 shows the results confirming the epithelial origin of mPAC-1, mPAC-1A, mPAC-2, mPAC-2A, mPAC-5, mPAC-5A, mPAC-6, mPAC-6A, mPAC-7, mPAC-18 and mPAC-25 and ductal origin of mPAC-6 and mPAC-25, and Figure 3.3 shows the phenotypical characterization of representative mPAC.

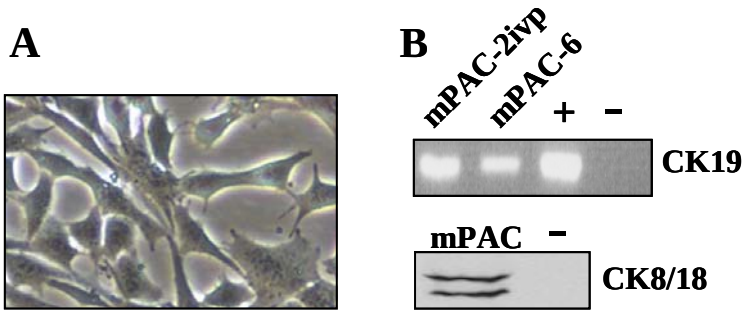

Figure 3.3. Phenotypical characterization of a murine pancreatic adenocarcinoma cell line, mPAC derived from a ductal adenocarcinoma of a TGF-α p53$^{-/-}$ mouse. (A) Phase-contrast microscopy of mPAC.
(B) Cytokeratin 19 expression (174 bp) of mPAC was analysed by RT-PCR and Cytokeratin 8/18 (52,5 kDa and 45,5kDa) expression by Western Blot.

CELL LINE	CK 8/18 (WESTERN BLOT)
mPAC-1	(+)
mPAC-1A	+
mPAC-2A	(+)
mPAC-2	+
mPAC-3	-
mPAC-4	-
mPAC-5	(+)
mPAC-5A	+
mPAC-6	+
mPAC-6A	+
mPAC-7	(+)
mPAC-7A	-
mPAC-8	-
mPAC-9	-
mPAC-10	-
mPAC-11	-
mPAC-12	-
mPAC-13	-
mPAC-18	(+)
mPAC-25	(+)
mPAC-2ivp	+

CELL LINE	CK 18 (RT-PCR)
mPAC-6	+
mPAC-25	+
mPAC-2ivp	+

CELL LINE	CK 19 (RT-PCR)
mPAC-6	+
mPAC-25	+
mPAC-2ivp	+

Table 3.1. Summary of Western Blot and RT-PCR analysis on Cytokeratin 8/18, 18 and 19 expression by different mPAC. (+) indicates weak signal for CK8/18 expression detected in Western Blot.

TGF-α transgenic mice express TGF-α under the regulation of the Elastase promotor to ensure that TGF-α overexpression is restricted to pancreatic acinar cells (Sandgren et al., 1990). To show that mPAC express the transgene TGF-α, a TGF-α-specific RT-PCR was performed (Fig. 3.4 A). The 158 bp product specific for TGF-α was detected in all mPAC tested. In Western Blotting, TGF-α expression (18 kDa) could only be detected in lysates of pancreata of TGF-α transgenic mice (Fig. 3.4 B) but not in mPAC.

To confirm that the TGF-α identified by RT-PCR is the overexpressed transgene, a second RT-PCR specific for the human Growth hormone (hGH), which is fused to the TGF-α transgene-construct, was performed (Fig 1.4 C). The 342 bp product specific for hGH could only be found in mPAC but in none of the irrelevant cell lines, such as Hepa1-6, RMA and NIH3T3, as tested by RT PCR.

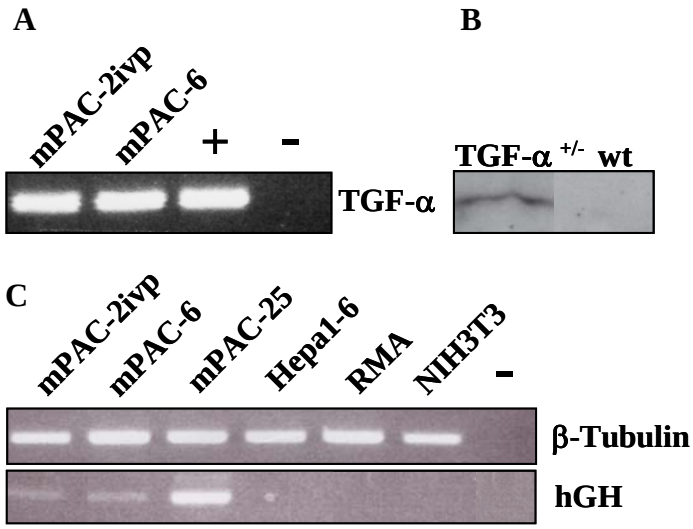

Figure 3.4. RT PCR/Western blot evaluation of transgene expression in mPAC. (A) TGF-α expression (158 bp) in mPAC was detected by RT PCR. (B) Western Blotting revealed a 18 kDa product for TGF-α expression only in pancreas of transgenic but not of non-transgenic wildtype littermates (wt). (C) RT-PCR showed expression of the human Growth Hormone (hGH: 342 bp), which is fused to the EL-TGF-α construct to produce TGF-α transgenic mice, in mPAC.

As shown in Fig. 3.5, FACS analysis using anti-H-2K^b and anti-I-A/I-E antibodies was performed to investigate MHC class I and class II expression on established tumor cell lines. All mPACs analysed expressed MHC class I but not class II. Individual mPAC showed variable levels of MHC class I expression, ranging from almost negative (mPAC-13) to highly positive (mPAC-6) cells.

mPAC that showed the best *in vitro* growth kinetics (data not shown) and were positive for Cytokeratin 8/18, Cytokeratin 19 and the trangene TGF-α were subjected to *in vivo* experiments.

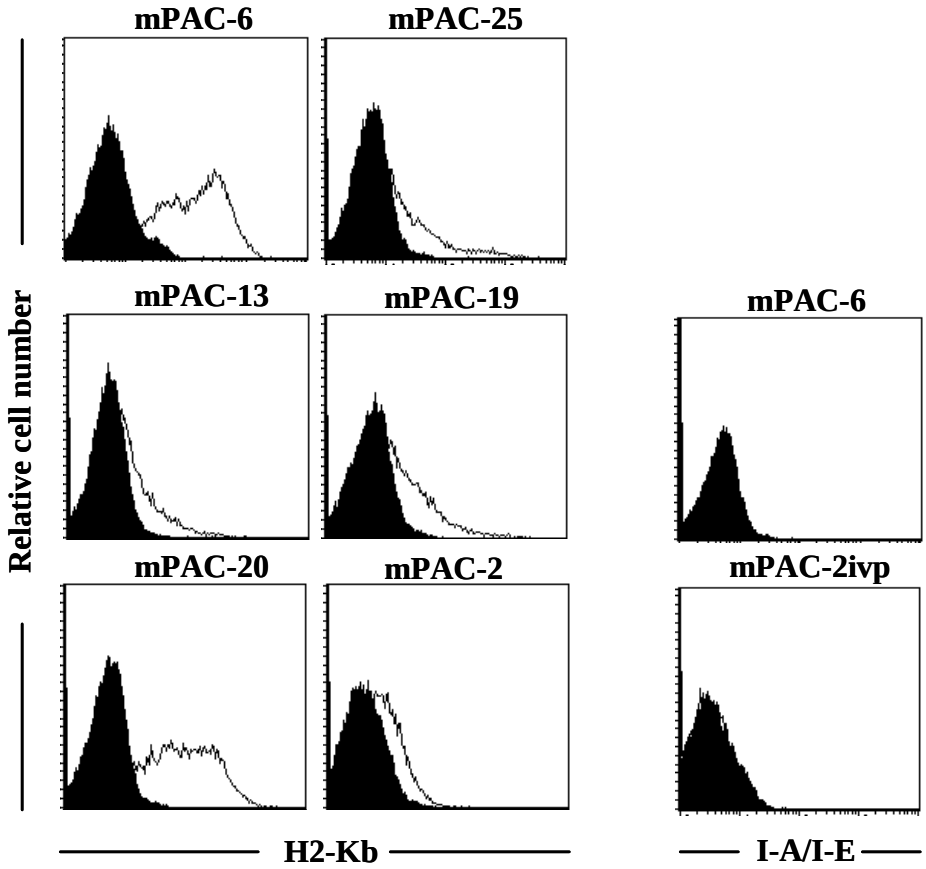

Figure 3.5. MHC expression of mPAC. mPAC showed diverse patterns of MHC class I expression. None of the cell lines tested showed expression of MHC class II. mPAC were analysed by FACS for surface protein expression using antibodies against MHC class I (anti-H2-K^b) and against MHC class II (anti-I-A/I-E). The filled histograms represent staining of mPAC with the appropriate isotype controls.

3.1.1.1 Tumor rejection after inoculation of mPAC

To analyse *in vivo* growth kinetics, mPAC derived from 3 different pancreatic tumors were subcutaneously injected into C57Bl/6 wildtype mice. After subcutaneous inoculation of 1 x 10^7 mPAC in C57Bl/6 wildtype mice, the tumor first started to grow during the first ten days but then regressed. 21 days after tumor injection no more tumor growth could be detected (Fig. 3.6). This reproducible regression of mPAC in immune competent mice was observed independently of the stage of MHC class I expression of the inoculated mPAC. In order to

look at the growth kinetics of mPAC in immunedeficient mice and to evaluate the immune mediation of this response, mPAC-2, mPAC-2A, mPAC-6, mPAC-6B were injected into RAG1-/- and nu/nu mice. Injection of mPAC in immune incompetent RAG1$^{-/-}$mice resulted in a slow but progressive outgrowth of the tumor. In RAG1$^{-/-}$ mice, there was also a slight regression in tumor growth observed between day 10-20 after mPAC inoculation, but the tumor was still detectable (2-3 mm tumor diameter). The reproducible outgrowth of different mPAC after this kind of growth stagnation in immune incompetent RAG1$^{-/-}$ mice versus complete regression after three weeks in immune competent wildtype mice clearly indicates, that the regression of mPAC was an immune regulated phenomenon. This immune dependent regression was further defined by the difference seen in the growth kinetics of mPAC in nu/nu mice compared to RAG1$^{-/-}$ mice (Fig. 3.6). In nu/nu mice, mPAC derived tumors regressed and stagnated until day 40, but then restarted to grow progressively to form lethal tumors as in RAG1$^{-/-}$ mice. However, compared to RAG1$^{-/-}$ mice, in nu/nu mice a more delayed tumor progression could be observed. RAG1$^{-/-}$ mice lack different lymphocyte populations that are important for tumor rejection than nu/nu mice. Thus, these data clearly indicate that the regression of mPAC derived tumors is T cell dependent, although a contribution of B cells and NKT cells could not be ruled out by these experiments.

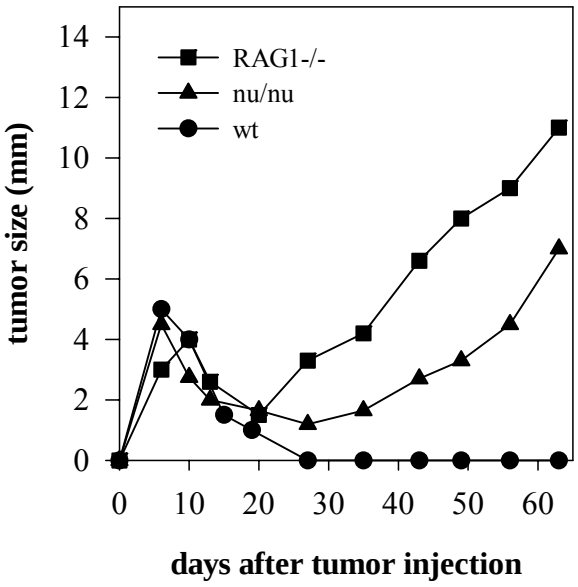

Figure 3.6. ***In vivo*** **growth kinetics of mPAC.** After s.c. inoculation into immune competent C57Bl/6 mice (circles), mPAC derived tumors grew during the first 10 days and then started to regress. 21 days after injection no more tumor growth was detectable in C57Bl/6 mice. Injection into immune deficient RAG1$^{-/-}$ (squares) and nu/nu (triangles) mice resulted in lagged but progressive outgrowth of the tumor. mPAC derived tumors grew faster in RAG1$^{-/-}$ (squares) than nu/nu (triangles) mice.

3.1.2 Cytotoxic activity of lymphocytes after immunization with mPAC

To further evaluate the immunogenicity of mPAC, C57Bl/6 mice were inoculated with irradiated mPAC. Immunization with mPAC but not with an irrelevant cell line (e.g. RMA) induced a strong cytotoxic T cell response against mPAC used as targets in CTL assay (Fig. 3.7 A). Two weeks after immunization, pooled splenocytes and lymph node cells from C57Bl/6 mice were analysed in CTL using MHC class I positive and MHC class I negative mPAC as targets at the effector to target (E:T) ratios indicated. In these CTL assays only MHC class I positive targets were lysed, which indicates that the detected cytotoxic response is $CD8^+$ T cell and not NK cell dependent (Fig. 3.7 B). Lymphocytes derived from mice immunized with RMA or from naïve control mice did not show mPAC-specific lysis.
Furthermore, mPAC-specific lysis could also be observed analysing splenocytes and lymph node cells of mPAC derived tumor bearing mice, i.e. in mice in which the mPAC derived tumor already regressed (data not shown).

To analyse, whether TGF-α might be the possible antigen recognized by the cytotoxic T cells after immunization with mPAC derived from mice overexpressing TGF-α, MHC class I positive target cells were transiently transfected with TGF-α-encoding plasmid DNA. No specific CTL response could be detected using B78H1K^bD^b/TGF-α or Hepa1-6/TGF-α as targets (Fig. 3.7 C). B78H1K^bD^b and Hepa1-6 transfected with a plasmid encoding an irrelevant antigen (GFP) served as controls. TGF-α expression of the target cells Hepa1-6/TGF-α and B78H1K^bD^b/TGF-α was confirmed in Western Blot (Fig. 3.7 D).

A second set of experiments was performed to rule out the possibility that TGF-α was the antigen: mPAC were injected into TGF-α transgenic mice, because these mice might exhibit tolerance against the potential antigen, since the antigen is expressed by mPAC. However, mPAC showed the same growth kinetics in TGF-α transgenic mice as in normal wildtype mice: The mPAC derived tumors grew and regressed within three weeks after tumor inoculation (data not shown).

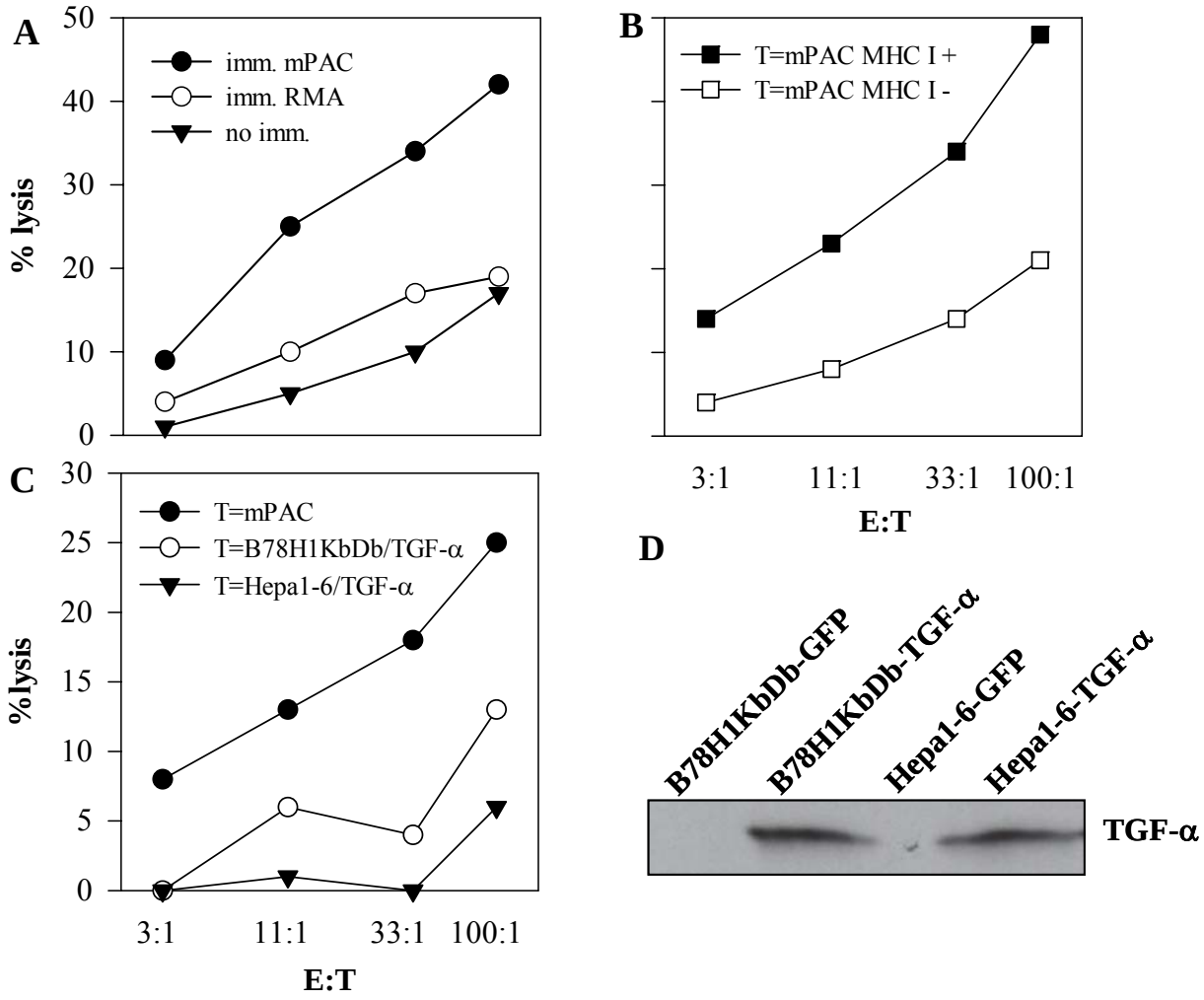

Figure 3.7. Cytotoxic activity in splenocytes and lymph node cells after immunization with mPAC. C57Bl/6 mice were immunized with irradiated mPAC or RMA cells. Two weeks after immunization pooled splenocytes and lymph node cells were restimulated *in vitro* for five days with mPAC and were subsequently analysed in standard ^{51}Cr CTL assays. (A) mPAC-specific lysis could only be observed after immunization with mPAC but not after immunization with irradiated RMA or in naïve mice. (B) Only MHC class I positive but not MHC class I negative mPAC-targets (T) were recognized in CTL. (C) No mPAC-specific cytotoxic activity was detected using TGF-α expressing B78H1K^{b}D^b or Hepa1-6 as targets (T). (D) TGF-α expression (15kDa) of B78H1K^{b}D^b and Hepa1-6 transiently transfected with TGF-α encoding plasmid DNA was verified by Western Blot.

3.1.2.1 mPAC-specific IFN-γ secretion in immunized mice

To further evaluate the observed mPAC-specific CTL response, the ability of $CD8^+$ T cells to secrete IFN-γ was analysed. Two weeks after immunization of C57Bl/6 mice with mPAC, splenocytes and lymph node cells were isolated, pooled and restimulated *in vitro* with mPAC. After restimulation, IFN-γ secretion was analysed performing IFN-γ capture assay. Using this method, the secreted IFN-γ is captured on the viable cells and subsequently labeled with a second IFN-γ specific antibody. FACS analysis of the cells counterstained with an anti-CD8 antibody revealed a significant IFN-γ secretion only in $CD8^+$ T cells derived from mice immunized with mPAC but not with control cell lines (e.g. RMA). After immunization with mPAC 4% of $CD8^+$ T cells secreted IFN-γ compared to 0,3% IFN-γ positive $CD8^+$ T cells after immunization with RMA (Fig. 3.8 A) or in naïve mice (data not shown). The detection of IFN-γ-secreting $CD8^+$ T cells supports the assumption that mPAC induced cytotoxicity is $CD8^+$ T cell dependent. Furthermore, this strong IFN-γ production detected by IFN-γ capture assay was confirmed in an independent experiment by intracellular cytokine staining (Fig. 3.8 B). Intracellular IFN-γ staining revealed a significant percentage of IFN-γ secreting $CD8^+$ cells after immunization with mPAC (0,6%) compared to 0,3% IFN-γ secreting $CD8^+$ cells after immunization with the control cell line RMA. These data show that the secreted IFN-γ in fact partly derived from $CD8^+$ T cells, but that also $CD8^-$ cells secreted significant amounts of IFN-γ.

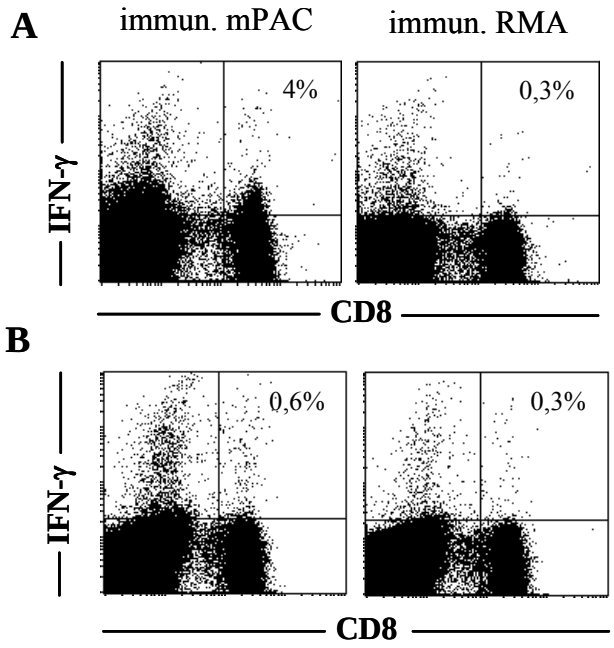

Figure 3.8: mPAC-specific INF-γ response. Lymphocytes from mPAC-immunized mice were in *in vitro* restimulated with mPAC and thereafter analysed for IFN-γ secretion. (A) IFN-γ capture assay revealed a strong IFN-γ secretion of $CD8^+$ T cells derived from mPAC immunized mice but not from mice immunized with RMA cells. (B) Intracellular IFN-γ staining confirmed IFN-γ production of $CD8^+$ T cells derived from mPAC immunized mice but not from mice immunized with RMA cells.

3.1.3 T cell infiltration of mPAC derived tumors

To investigate tumor infiltrating lymphocytes, immunohistological analysis and FACS analysis of mPAC derived tumors at day seven after subcutaneous tumor inoculation were performed. Immunohistological analysis of frozen tumor sections revealed that these tumors were highly infiltrated with $CD4^+$ and $CD8^+$ T cells. Both T cell populations could also be detected by FACS analysis of tumor infiltrating lymphocytes after Collagenase/Dispase digestion of the tumors . Significant populations of 17% $CD4^+$ T cells and 6% $CD8^+$ T cells could be detected (Fig. 3.9).

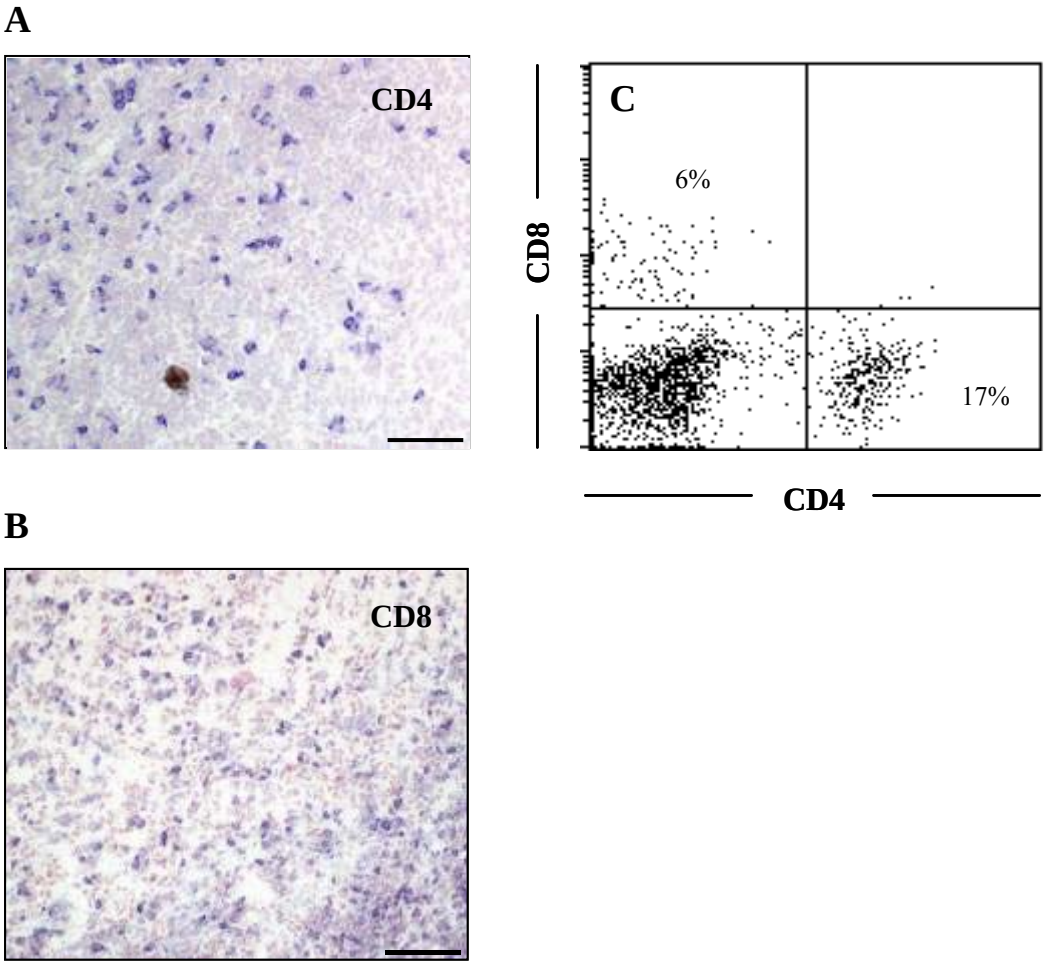

Figure 3.9. Tumor infiltration by T cells. Seven days after s.c. injection of mPAC immunohistological analysis of frozen sections revealed infiltration of $CD4^+$ (A) and $CD8^+$ (B) T cells (bar, 100 µm). (C) FACS analysis of tumor infiltrating lymphocytes at day seven after mPAC injection confirmed the tumor infiltration with $CD4^+$ (6%) and $CD8^+$ (19%) T cells. These results are representative for two independent experiments.

3.1.4 Induction of mPAC-specific humoral responses

To examine the contribution of B cells in the rejection of mPAC derived tumors, three weeks after subcutaneous inoculation of mPAC, sera (1:50 dilution) from C57Bl/6 mice were analysed for mPAC-specific antibodies. FACS analysis of the sera revealed that C57Bl/6 mice bearing mPAC derived tumors exhibit a humoral response against mPAC, but not

against a control cell line (Fig. 3.10). Sera taken from mice before mPAC inoculation served as control (filled histograms).

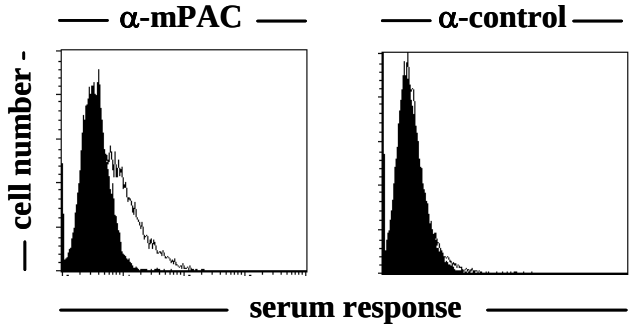

Figure 3.10. mPAC induced humoral response after s.c. injection into C57-Bl/6 mice. Three weeks after injection of mPAC, antibodies against mPAC but not against a control cell line could be identified in sera of mice (1:50 dilution) as shown in histogram overlay. Filled histogram represents analysis of sera taken before injection of mPAC.

3.1.5 Histological analysis of pancreatic tumors of TGF-α Trp53$^{-/-}$ mice at different time points

Histological analysis of pancreatic tumors of TGF-α Trp53$^{-/-}$ mice at different time points revealed that at 6 weeks of age the pancreas was already highly enlarged, but showed to a large extend a normal morphology. The majority of the tissue was not yet highly proliferating, however hypertrophic acini, which sometimes showed a pleomorphic phenotype, could already be observed. Some areas of proliferating cells showed irregular outlines but with "pushing margins" towards the adjacent parenchyma. In these areas the cellular morphology was altered insofar as they had hyperchromatic nuclei and exhibited a loss of nuclear polarity. Mitosis, necrosis and apoptosis were rare, but mitosis were not atypical (Fig. 3.11 A-C).
In 9-week-old mice the acinar structure of the pancreas was already lost in most of the lesions. The cells started to grow in layers ("budding off"). They were characterized by an increase in the nucleus-to-cytoplasm ratio. Bizarre giant nuclei and severe dysplasia were found. The number of areas with proliferating cells was higher and in contrast to 6-week-old mice there was also an increase of proliferation (increased mitosis index) and more interstitial fibrosis (data not shown).

In 12-week-old TGF-α Trp53$^{-/-}$ mice, the regular acinus structures were mostly lost. The tissue was replaced by highly proliferating trabecular and tubular structures showing vesicular nuclei and in part prominent nuclei. Multiple mitoses were present, many of them atypical. Moreover, there was a marked increase of apoptotic bodies. In some areas, microcystic transformation occured. The histomorphological picture corresponded to a highly differentiated carcinoma (Fig. 3.11 D-F).

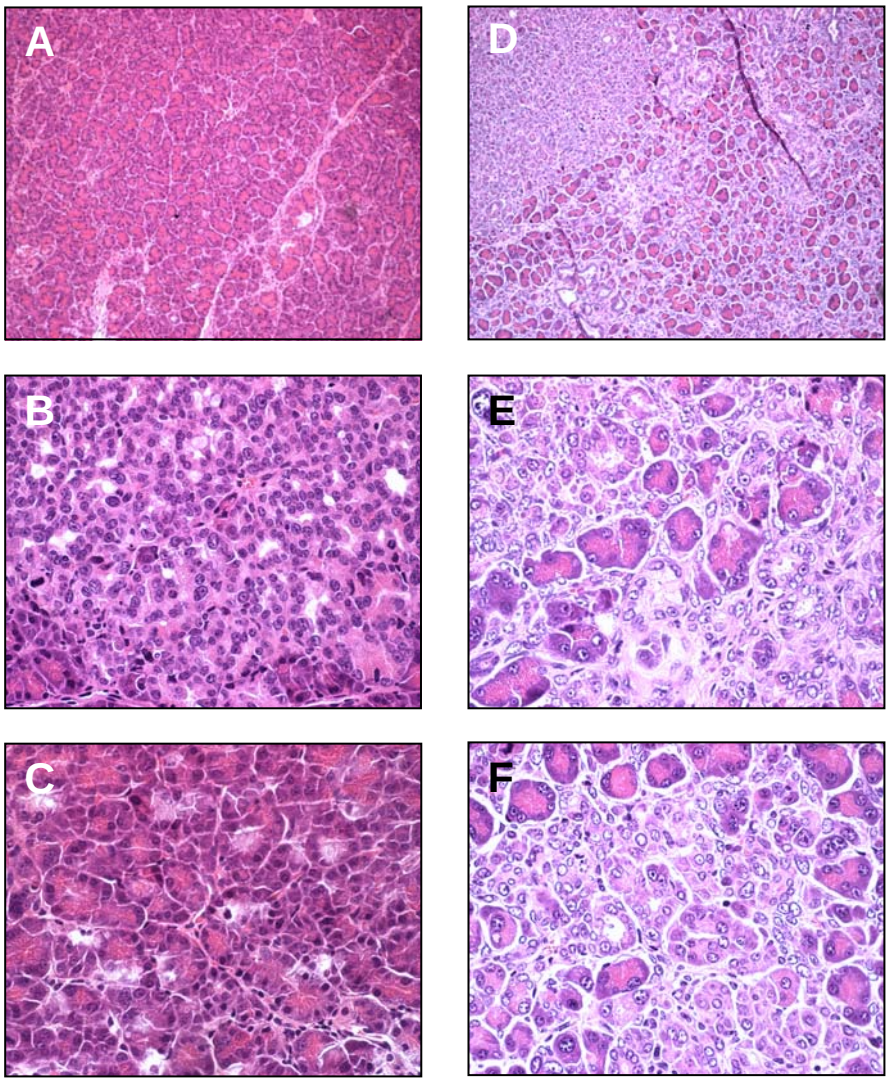

Figure 3.11. Histological analysis of representative pancreatic tumors of TGF-α Trp53$^{-/-}$ mice of different ages. The pancreas of 6-week-old TGF-α Trp53$^{-/-}$ mice showed to a large extent a normal morphology (A). Only in some irregularly proliferating areas with "pushing margins" the acinar morphology was already altered (B,C). The pancreas of 12-week-old TGF-α Trp53$^{-/-}$ mice showed the morphological character of a highly differentiated carcinoma with lost acinar structure and formation of duct-like tubular complexes and vesicular nuclei (D-F). Each tissue was stained with hematoxilin and eosin (original magnification 100x [A,D] and 400x [B,C,E,F]).

3.1.6 Normal distribution of lymphocyte populations in TGF-α Trp53$^{-/-}$ mice

To evaluate whether TGF-α Trp53$^{-/-}$ mice develop a normal immune system, lymphocyte populations of TGF-α Trp53$^{-/-}$ mice were analysed in comparison to non-transgenic littermate control mice. The percentage of NK cells, Dendritic cells, Macrophages and B and T cells were evaluated in TGF-α Trp53$^{-/-}$ mice and in non-transgenic littermates. FACS analysis revealed that TGF-α Trp53$^{-/-}$ mice have normal numbers of DX5^{+} NK cells (2,5% compared to 2,5%), CD11c^{+}MHC class II^{+} Dendritic cells (2,5% compared to 2,1%), CD11b^{+}MHC class II^{+} Macrophages (2,9% compared to 2,6%), CD19^{+}B220^{+} B cells (61,7% compared to 56,2%), CD8^{+} (8,6% compared to 10%) and CD4^{+} T cells (30,5% compared to 33,2%) and

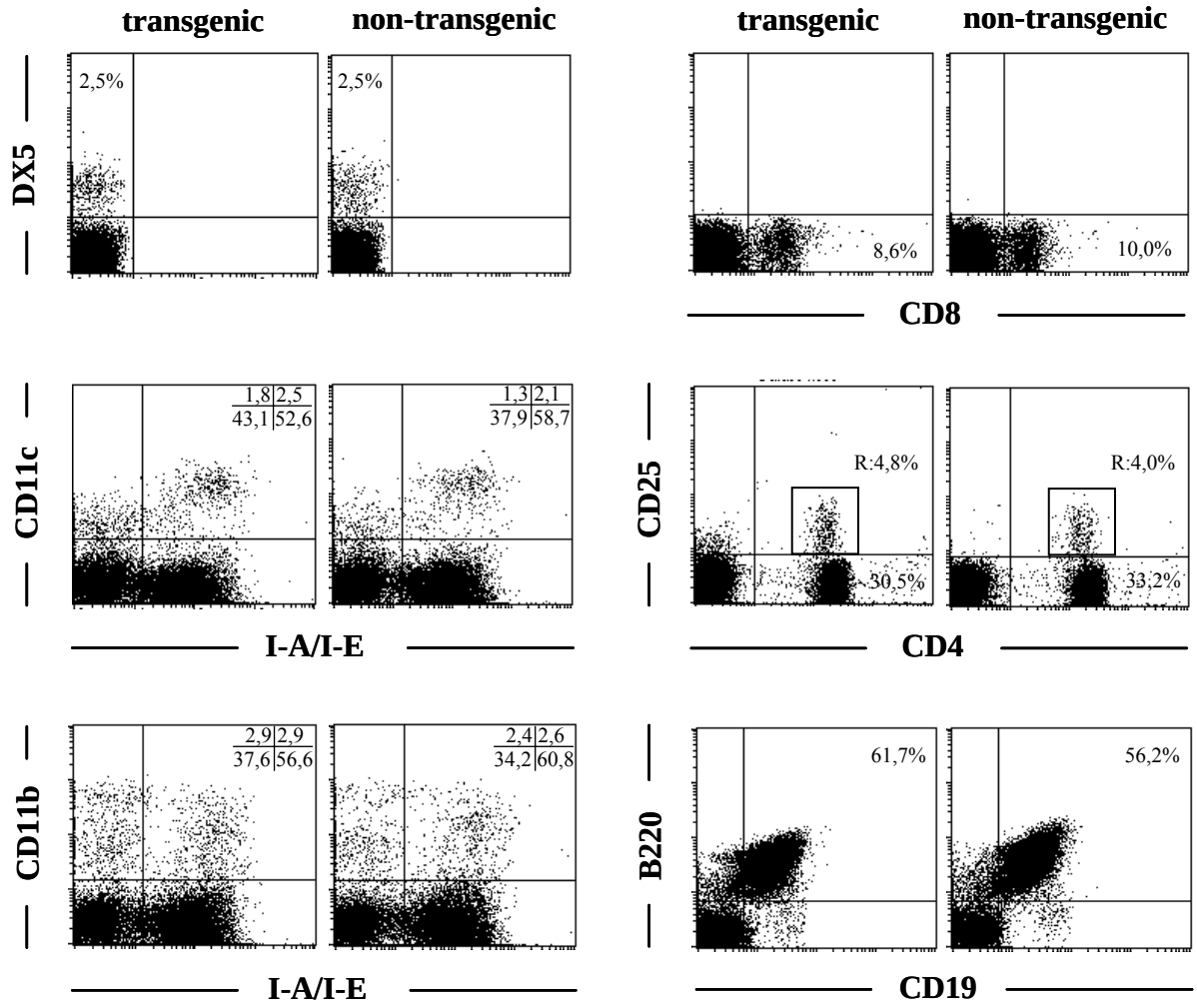

Figure 3.12. Analysis of lymphocyte populations in TGF-α Trp53$^{-/-}$ mice. Lymphocytes from TGF-α Trp53$^{-/-}$ and non-transgenic littermate control mice were isolated and single cell suspensions were stained for the distinct lymphocyte populations and analysed by FACS. No significant difference concerning the distribution of the specific lymphocyte populations could be seen comparing transgenic (TGF-a Trp53$^{-/-}$) and non-tansgenic mice.

$CD4^+CD25^+$ regulatory T cells (4,8% compared to 4,0%). These data indicate that TGF-α $Trp53^{-/-}$ mice exhibited a normal development and distribution of the distinct lymphocyte populations compared to non-transgenic littermate control mice.

3.1.7 Analysis of immune responses in TGF-α $Trp53^{-/-}$ mice

All experiments described above demonstrated that mPAC were highly immunogenic tumors, inducing tumor-specific humoral and cellular immune responses in wildtype mice. However, in the past it has been shown that tumors can impair immune responses. In addition, not all tumors induce specific immune responses, but rather tumor specific tolerance. Therefore, we next analysed tumor specific immune responses in TGF-α $Trp53^{-/-}$ mice. Additionally, we tested mice with premalignant lesions.

Analysis of a detectable immune response in TGF-α $Trp53^{-/-}$ mice against the highly immunogenic cell line mPAC revealed an IFN-γ response only in tumor bearing TGF-α $Trp53^{-/-}$ mice (Fig. 3.13). Pooled splenocytes and lymph node cells of pancreatic tumor bearing 12-week-old TGF-α $Trp53^{-/-}$ mice, their age-matched heterozygous littermate control mice and of 6-week-old TGF-α $Trp53^{-/-}$ mice and their age-matched heterozygous littermate control mice were restimulated *in vitro* with mPAC and subsequently analysed for IFN-γ secretion. FACS analysis of IFN-γ capture assay revealed a reproducible significant number of IFN-γ secreting $CD8^+$ T (1%) cells only in 12-week-old TGF-α $Trp53^{-/-}$ mice, which already developed malignant tumors, but neither in 6-week-old TGF-α $Trp53^{-/-}$ mice (0,2%), which did not yet develop tumors, nor in heterozygous littermates (0,2%). These data clearly indicate that the induction of a $CD8^+$ T cell response against the highly immunogenic cell line mPAC occurs only in association with the malignant pancreatic tumors. The immune response is not induced before the tumor arises. Additionally, this $CD8^+$ dependent IFN-γ secretion was also confirmed in an independent experiment by intracellular staining for IFN-γ (data not shown).

A CTL response against mPAC or target cells transfected with TGF-α encoding plasmid DNA could not be observed in naive TGF-α $Trp53^{-/-}$ mice of any age (data not shown),

indicating that the $CD8^+$ T cells in TGF-α $Trp53^{-/-}$ mice were only partially activated to secrete IFN-γ, but not to lyse the target cells. However, independent of their age, TGF-α $Trp53^{-/-}$ mice were able to reject transplanted mPAC derived tumors in the same manner as non-transgenic wildtype mice.

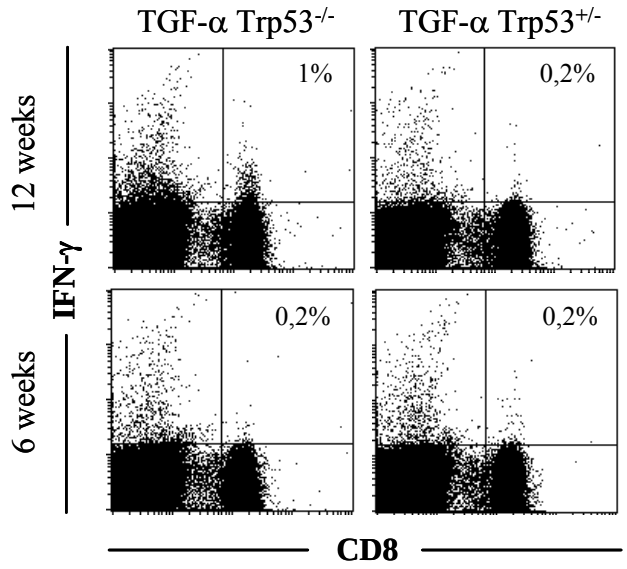

Figure 3.13. Analysis of IFN-γ secretion of $CD8^+$ T cells. Pooled splenocytes and lymph node cells of 12-week-old TGF-α $Trp53^{-/-}$ and TGF-α $Trp53^{+/-}$ mice and of 6-week-old TGF-α $Trp53^{-/-}$ and TGF-α $Trp53^{+/-}$ mice were restimulated *in vitro* with mPAC and analysed by IFN-γ capure assay. FACS analysis revealed that only mice that already bear pancreatic tumors show IFN-γ secretion of $CD8^+$ T cells.

To analyse the tumors for the presence of inflammatory cytokines, pancreatic tumors of TGF-α $Trp53^{-/-}$ mice at different ages (6 and 12 weeks) and mPAC derived tumors (d 7 after s.c. inoculation into C57Bl/6 wildtype mice) were explanted, cut into small pieces and incubated *in vitro*. After over night incubation, culture supernatants were harvested and analysed for several cytokines in Cytometric Bead Array (CBA) and ELISA (Fig. 3.14).

Analysis of the tumor derived supernatants by CBA revealed no IFN-γ in any of the autochthonous tumors analysed, but a significant IFN-γ production in explanted mPAC derived tumors. These results were confirmed by ELISA specific for IFN-γ (data not shown).
TNF-α was only found in very low amounts in the supernatants of tumors derived from 6-week-old TGF-α Trp53$^{-/-}$ mice, but at six-fold higher levels in the supernatants of mPAC derived tumors.
Consistent with this inflammatory phenotype, also IL-12 was detected in the supernatant of mPAC derived tumors. Slight levels of IL-12 could also be detected in the culture supernatants derived from the autochthonous pancreatic tumors. None of the analysed tumor derived supernatants was positive for the inhibitory cytokine IL-10.
Similarly to these results, no tumor infiltrating lymphocytes could be detected in histological analysis of pancreatic tumors of TGF-α Trp53$^{-/-}$ mice at any stage of tumor development.

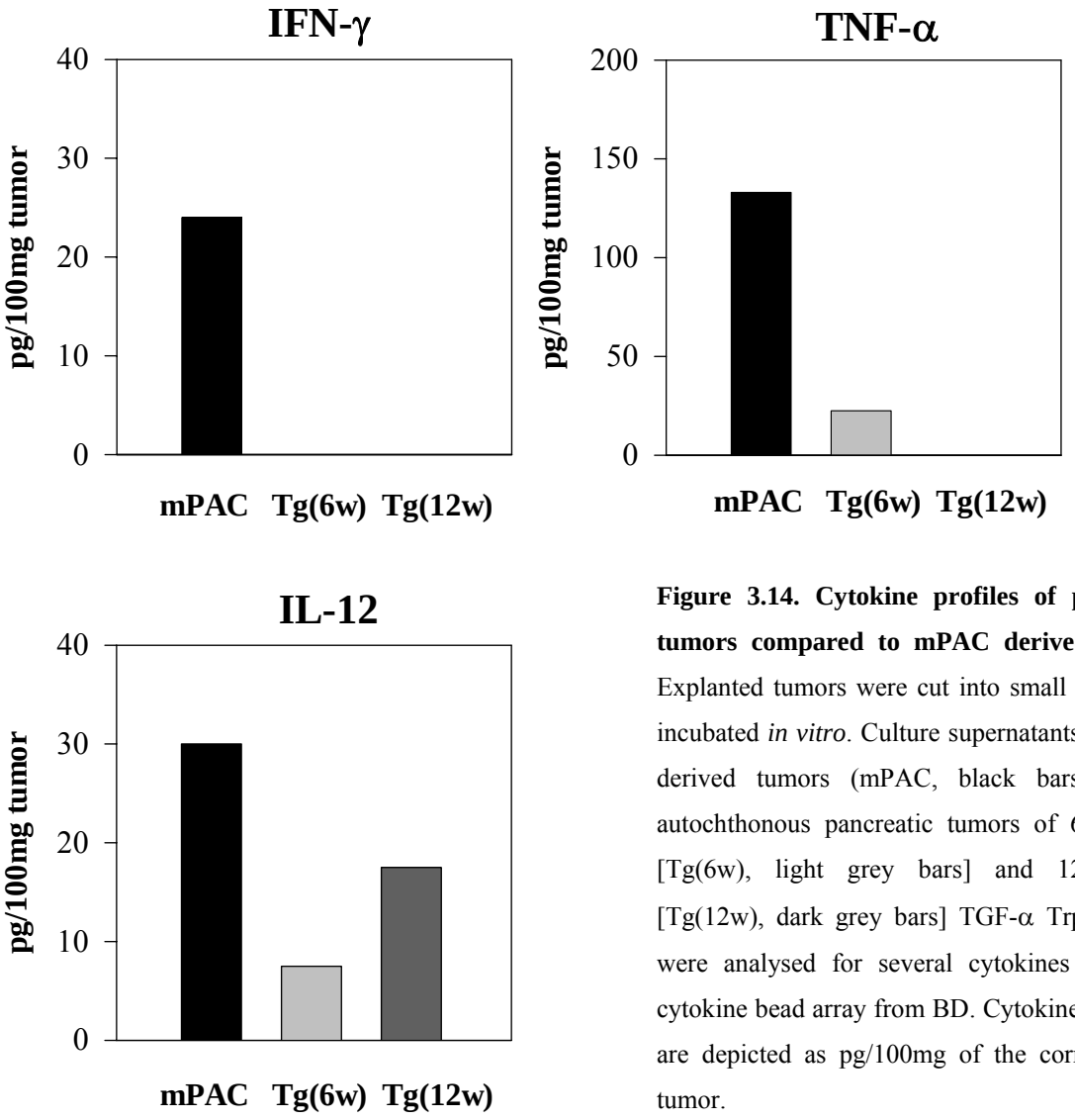

Figure 3.14. Cytokine profiles of pancreatic tumors compared to mPAC derived tumors. Explanted tumors were cut into small pieces and incubated *in vitro*. Culture supernatants of mPAC derived tumors (mPAC, black bars) and of autochthonous pancreatic tumors of 6-week-old [Tg(6w), light grey bars] and 12-week-old [Tg(12w), dark grey bars] TGF-α Trp53$^{-/-}$ mice were analysed for several cytokines using the cytokine bead array from BD. Cytokine quantities are depicted as pg/100mg of the corresponding tumor.

To further evaluate the tumor-specific immune response in TGF-α Trp53$^{-/-}$ mice, sera of 6-week-old and 12-week-old mice and their age-matched heterozygous littermates were analysed for mPAC-specific antibodies. A humoral response against mPAC, but not against a control cell line in the sera of four of nine 12-week-old and a weaker response in the sera of three of seven 6-week-old TGF-α Trp53$^{-/-}$ mice could be detected (Fig. 3.15). No antibody response could be detected in the sera of heterozygous littermate control mice (0 of 4). Increased levels of anti-mPAC antibodies in the sera of TGF-α Trp53$^{-/-}$ mice as tumor progresses corresponded to the increased levels of IFN-γ secretion detected by IFN-γ capture assay .

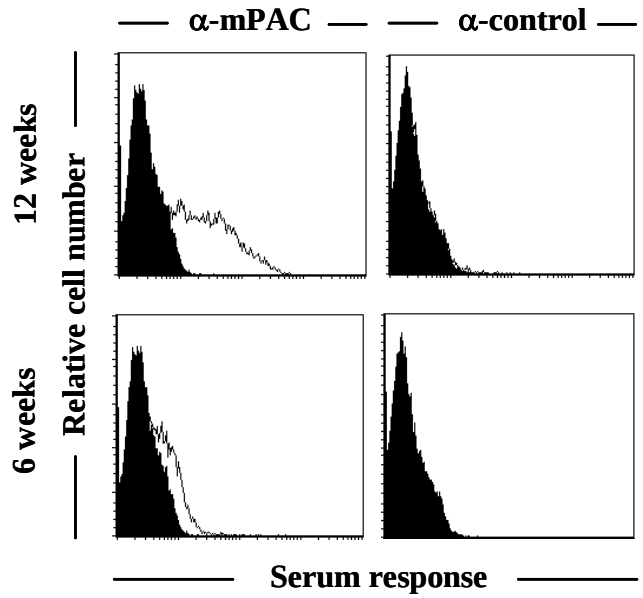

Figure 3.15. Humoral responses against mPAC in TGF-α Trp53$^{-/-}$ mice. Antibodies against mPAC but not against a control cell line could be identified in sera of tumor bearing 12-week-old TGF-α Trp53$^{-/-}$ mice (1:50 dilution) as shown in histogram overlay. 6-week-old TGF-α Trp53$^{-/-}$ mice exhibit only very slight antibody responses against mPAC.

3.2 Selection of tumor escape variants mPACivp

Murine pancreatic adenocarcinoma cell lines originated from pancreatic tumors of TGF-α $Trp53^{-/-}$ mice on C57Bl/6 background did not grow progressively when inoculated subcutaneously into syngenic immune competent mice. The tumors grew progressively, even though with a short delay, when injected into nu/nu and $RAG1^{-/-}$ mice, proving the malignant potential of these cell lines.

After injection into immune competent mice, mPAC derived tumors regressed after 21 days, but in a minority of the mice, progressively growing tumors were observed 2-4 months after inoculation. These rare progressor tumors were isolated from normal C57Bl/6 mice when they had grown to a size of 10-15 mm in diameter, homogenized and taken into culture. Cell lines were established from these tumors and were named mPACivp (*in vivo* passaged). Injection of $1x10^7$ mPAC-2ivp cells produced progressively growing tumors in 100% of the immune competent syngenic mice (Fig. 3.16), thereby demonstrating the heritable nature of the progressive growth behaviour of the newly isolated tumor mPAC-2ivp.

However, injection of the escape variant mPAC-2ivp into nu/nu mice did not result in faster outgrowth of the tumors compared to immune competent mice (data not shown).

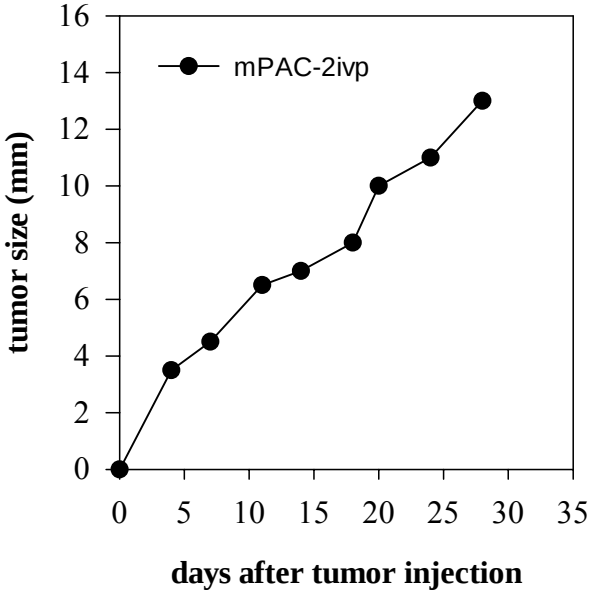

Figure 3.16. mPAC-2ivp grew out to form lethal tumors. After reisolation and establishment of this rare new cell line, s.c. injection of mPAC-2ivp resulted in progressive tumor growth in 100% of immune competent C57Bl/6 mice.

To prove that mPAC-2ivp and the highly immunogenic mPAC-6 derived from the same pancreatic tumor of a TGF-α Trp53$^{-/-}$ mouse, the genes coding for the human growth hormone (hGH) and for p53 were used as lineage-specific markers. Isolation of genomic DNA and subsequent analysis by PCR revealed the presence of the TGF-α/hGH fusion gene and the absence (disruption) of the p53 gene in the original mPAC-6 as well as in the variant mPAC-2ivp (Fig. 3.17).

Further analysis showed that mPAC-2ivp expresses Cytokeratins 8/18 and 19 as markers for epithelial and ductal origin and the transgene TGF-α and hGH, respectively (Fig.3.3 and Fig.3.4). These data clearly indicate, that mPAC-2ivp and mPAC-6 have the same origin.

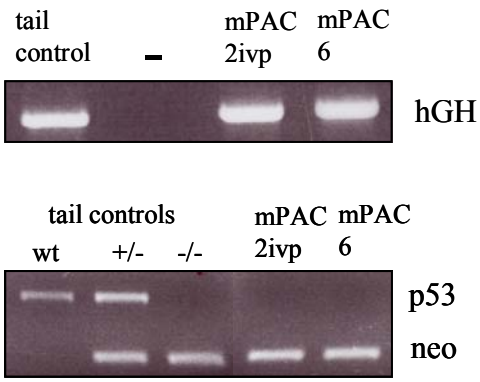

Figure 3.17. mPAC-2ivp exhibited the same lineage markers as its original cell line mPAC-6. Analysis of genomic DNA by PCR detected the hGH gene but not the by the insertion of neo disrupted p53 gene in mPAC-2ivp and in mPAC-6. These results matched with the genotype of TGF-α Trp53$^{-/-}$ mice the cell lines derived of.

3.2.1 MHC class I expression and tumor progression

Analysis of MHC class I expression by FACS showed that mPAC-2ivp has very low MHC class I expression, compared to the original mPAC (Fig. 3.18 A and Fig. 3.5). Induction of MHC class I expression by addition of IFN-γ to the cell culture medium revealed that mPAC-2ivp has not lost but down-regulated MHC class I expression, because after incubation with IFN-γ 100% of the cells were highly positive for MHC class I expression. (Fig.3.18 A).

To analyse, whether down-regulation of MHC class I expression might be the reason for the escape of immune destruction, a MHC class I positive clone of mPAC-2ivp was used for

further experiments. This clone exhibited stable MHC class I expression *in vitro* (Fig. 3.18 B) without addition of IFN-γ and showed similar *in vivo* growth kinetics as mPAC-2ivpMHC I^- after s.c. inoculation into C57Bl/6 mice (Fig.3.18 C). The tumors were isolated, collagenase/dispase digested and the cells were subsequently analysed by FACS for MHC class I expression. "*Ex vivo*" FACS analysis of mPAC-2ivpMHC I^+ and mPAC-2ivpMHC I^- demonstrated that the MHC class I positive clone mPACivpMHC I^+ did not down-regulate MHC class I expression *in vivo* (Fig. 3.18 B). These data support the idea that the *in vivo* tumor progression of mPAC-2ivp is independent of MHC class I expression.

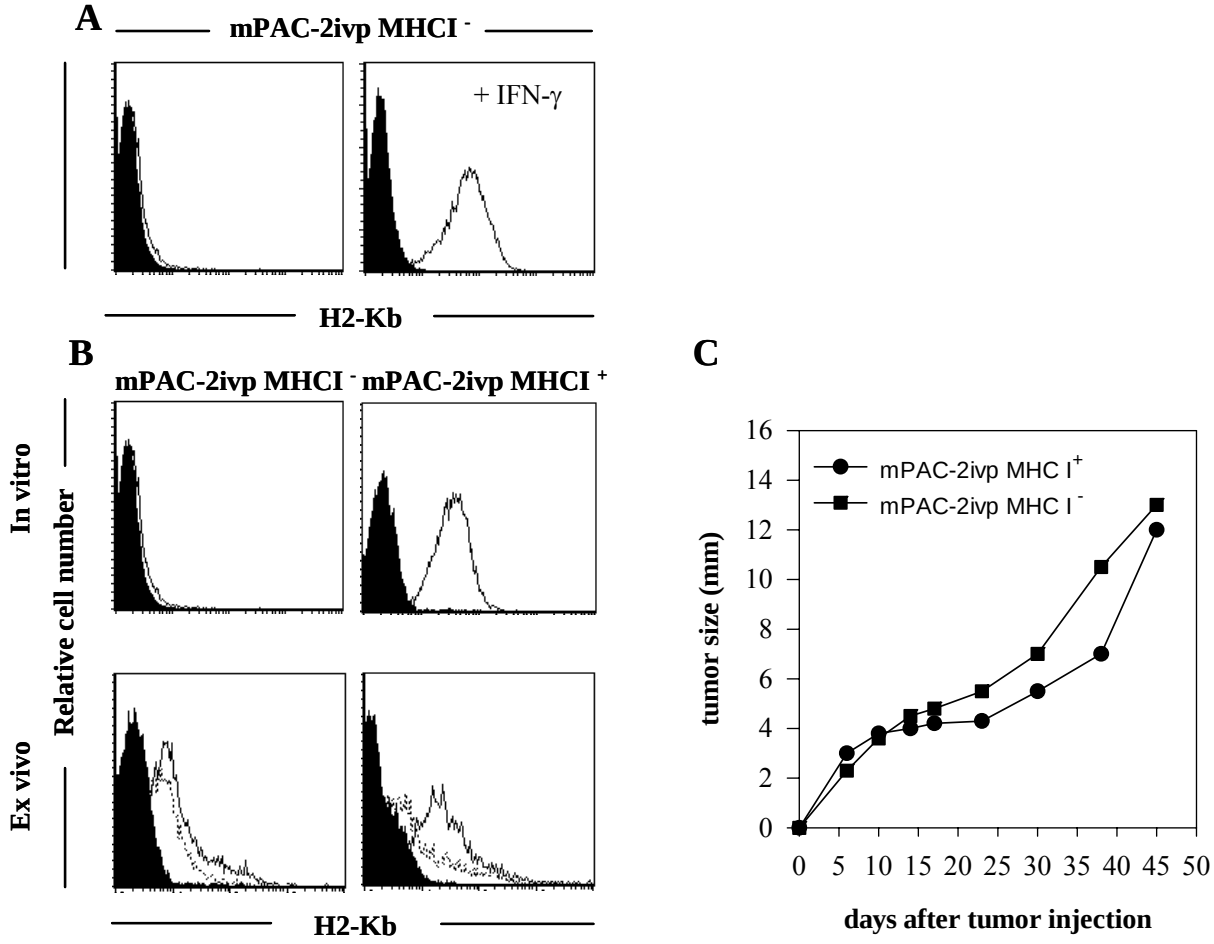

Figure 3.18. Progressive *in vivo* growth of mPAC-2ivp was independent of MHC class I expression. (A) FACS analysis of mPAC-2ivp for surface expression of MHC class I revealed that MHC I down-regulation could be reversed by addition of IFN-γ to the cell culture medium (100U/ml for 24h). (B) Cloning of mPAC-2ivp under limiting dilution conditions results in the generation of the clone mPAC-2ivpMHC I^+ that exhibited a stable and high expression MHC class I *in vitro* and *ex vivo* after tumor explantion. Dotted lines represent isotype control. (C) Progressive *in vivo* growth of mPACivp was independent of MHC class I expression.

3.2.2 Injection of mPAC-6 and mPAC-2ivp into the same mouse to evaluate cross-protection

To find out, whether mPAC-6 and mPAC-2ivp are immunologically related, mPAC-6 and mPAC-2ivp were injected s.c. into the same C57Bl/6 mouse (Fig. 3.19). If the mice were rechallenged after 10 days with mPAC-6, the mice were protected against growth of the second challenge with mPAC-6 (Fig. 3.19 A). This is another indication for the immunogenic phenotype of mPAC-6. Challenge of the mice with mPAC-2ivp after previous injection of mPAC-6 did not impair the progressive outgrowth of mPAC-2ivp (Fig. 3.19 B). In addition, to analyse whether previous injection of mPAC-2ivp facilitates the growth of mPAC-6 as progressive tumor, the reverse experiment was done. However, challenging C57Bl/6 mice with mPAC-6 after previous injection of mPAC-2ivp resulted in the same growth kinetics of mPAC-6 as seen in untreated mice: The mPAC-6 derived tumor grew during the first ten days and then started to regress (Fig. 3.19 C).

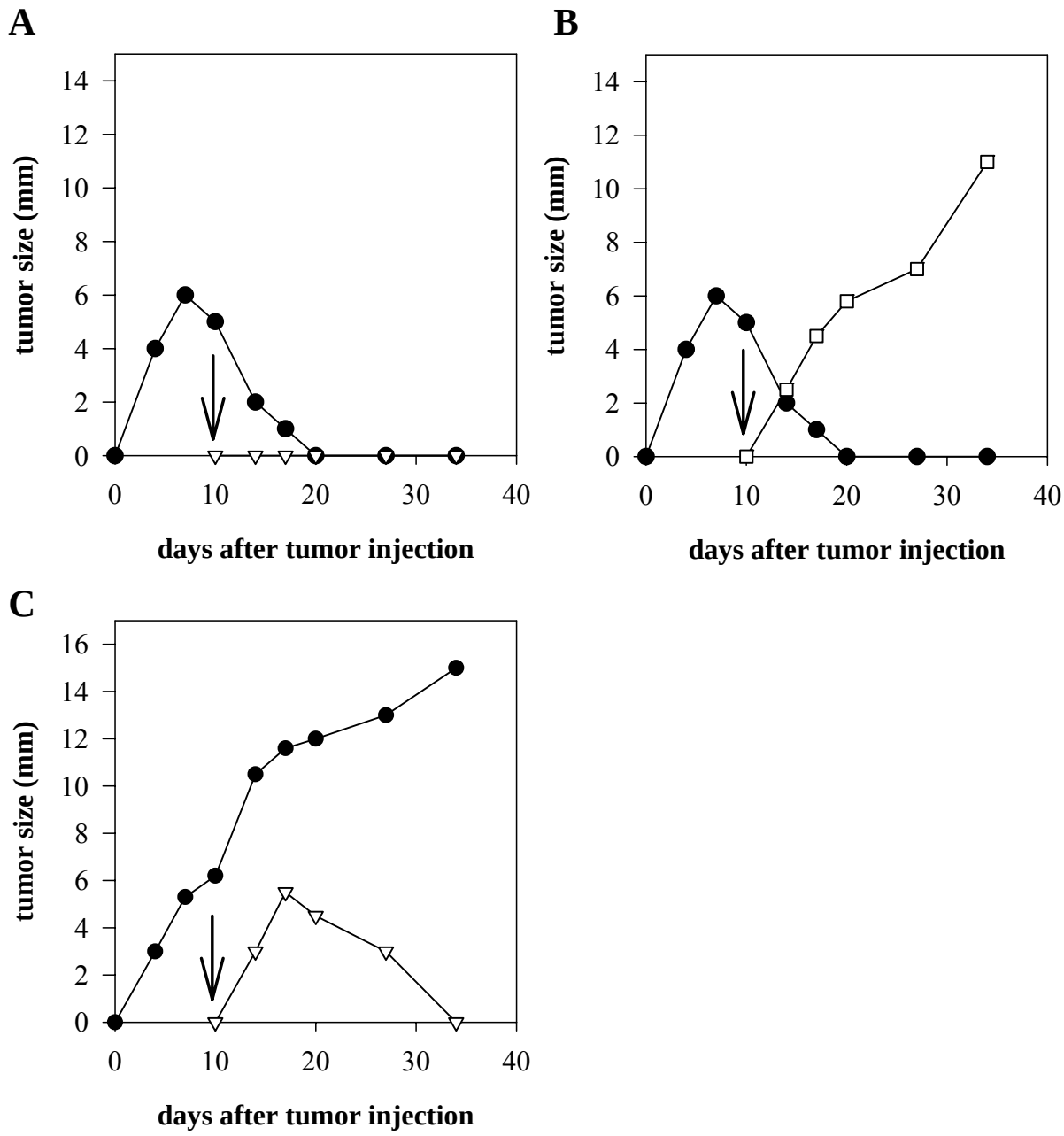

Figure 3.19. Inoculation of mPAC-6 and mPAC-2ivp into the same mouse. (A) Repeated injection of mPAC-6 after 10 days into the opposite hind flank of the same mouse immunized the mouse against the second challenge. (B) Preinjection with the immunogenic mPAC-6 did not prevent the progressive growth of mPAC-2ivp. (C) mPAC-2ivp tumor bearing mice were not tolerant to the outgrowth of mPAC-6.

3.2.3 Immune responses against mPAC-6 and mPAC-2ivp

3.2.3.1 mPAC-6- and mPAC-2ivp-specific cytotoxicity in immunized mice

To further investigate whether mPAC-6 and mPAC-2ivp share similar T cell antigens, CTL assays using both variants were performed. Two weeks after immunization with irradiated mPAC-6 or mPAC-2ivp, splenocytes and lymph node cells from C57Bl/6 mice were isolated, pooled and restimulated *in vitro* either with mPAC-6 or with mPAC-2ivp MHC I^+. After five days of *in vitro* restimulation, mPAC-6- and mPAC-2ivp-specific lysis was analysed in standard ^{51}Cr CTL assays. The assays were performed using the cell line mPAC-2ivpMHC I^+ (MHC class I was up-regulated by adding IFN-γ to the cell culture medium) as restimulator and target and not the MHC I^+ clone to ensure the availability of all possible antigens. A diagram of the experimental procedure is depicted below (Fig. 3.20).

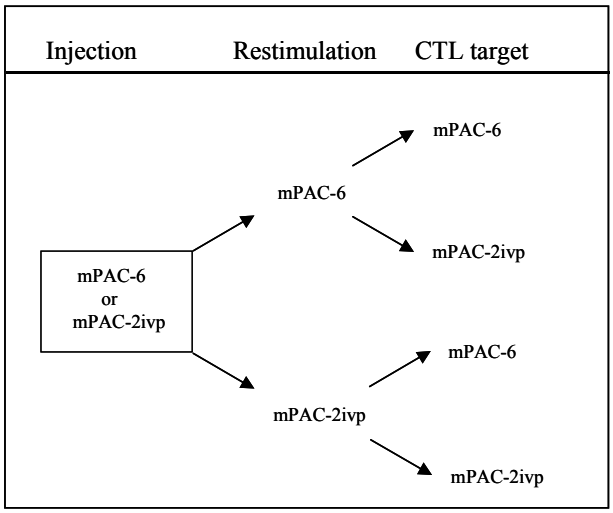

Figure 3.20: Experimental set up for the performance of CTL assays.

CTL assays performed with mPAC-6 or mPAC-2ivp as restimulators and targets revealed cross-reactivity of the two variants. A mutual and clearly MHC class I dependent cytotoxic activity could be shown for almost all conditions analysed. To exclude that the observed mutual CTL lysis was an *in vitro* artefact, lymphocytes of mice immunized with an irrelevant tumor cell line, RMA, served as control. The results of the performed CTL assays indicate that mPAC and mPACivp share similar T cell antigens. Mice could be immunized with mPAC-6 and cross-restimulation of lymphocytes derived from these mice with mPAC-2ivp

resulted in subsequent lysis of mPAC-2ivp (mPAC-2ivp → mPAC-2ivp) or mPAC-6 (mPAC-2ivp → mPAC-6), furthermore they can be restimulated with mPAC-6 to subsequently lyse mPAC-2ivp (mPAC-6 → mPAC-2ivp).

Lymphocytes isolated from mice immunized with mPAC-2ivp could be restimulated with mPAC-2ivp to lyse mPAC-2ivp (mPAC-2ivp → mPAC-2ivp) and also to lyse mPAC-6 (mPAC-2ivp → mPAC-6), but cross-restimulation with mPAC-6 did only result in weak lysis of mPAC-6 (mPAC-6 → mPAC-6) and in no significant lysis of mPAC-2ivp (mPAC-6 → mPAC-2ivp) compared to control mice (Fig. 3.21 and Tab. 3.2).

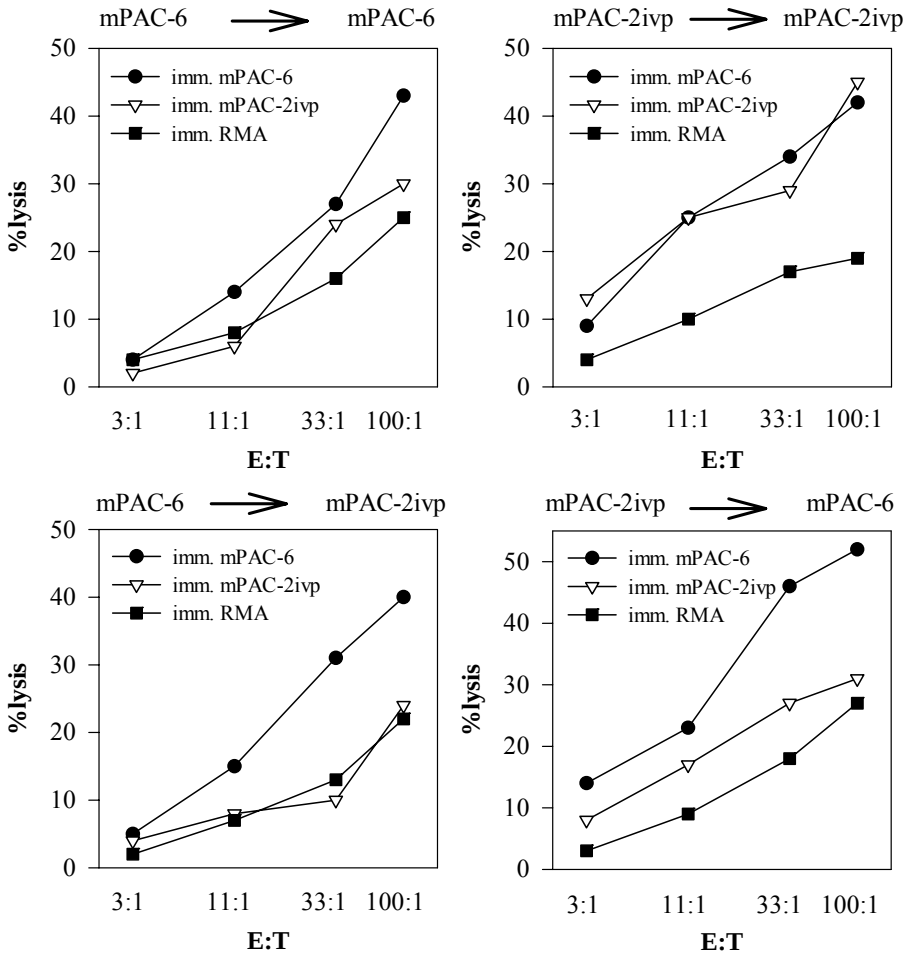

Figure 3.21. Performance of CTL assays showed mutual CTL lysis after immunization with mPAC-6 or with mPAC-2ivp. C57Bl/6 mice were immunized with irradiated mPAC-6 (filled circles), mPAC-2ivp (open triangles) or with control RMA (filled squares). Isolated lymphocytes were *in vitro* restimulated with mPAC-6 or mPAC-2ivp and tested in CTL against mPAC-6 or mPAC-2ivp used as targets. The results obtained after restimualtion with mPAC-6 and using mPAC-6 as a target (mPAC-6 → mPAC-6), restimulation with mPAC-2ivp and using mPAC-2ivp as target (mPAC-2ivp → mPAC-2ivp), restimulation with mPAC-6 and using mPAC-2ivp as target (mPAC-6→ mPAC-2ivp) and restimulation with mPAC-2ivp and using mPAC-6 as target (mPAC-2ivp → mPAC-6) are summarized in Table 3.2.

INJECTION	RESTIMULATION	CTL TARGET	LYSIS
mPAC-6	mPAC-6	mPAC-6	+
		mPAC-2ivp	+
	mPAC-2ivp	mPAC-6	+
		mPAC-2ivp	+
mPAC-2ivp	mPAC-6	mPAC-6	+/-
		mPAC-2ivp	-
	mPAC-2ivp	mPAC-6	+
		mPAC-2ivp	+

Table 3.2: Summary of CTL Assay results.

3.2.3.2 mPAC-6- and mPAC-2ivp-specific IFN-γ secretion in immunized mice

In order to support results obtained by cytotoxicity assays, we also performed IFN-γ-specific ELISA after 48h of restimulation. Comparable to the CTL results, significant amounts of IFN-γ could only be detected in supernatants of lymphocytes derived from C57Bl/6 mice immunized with mPAC-6 or mPAC-2ivp but not in those supernatants of lymphocytes derived from control mice.

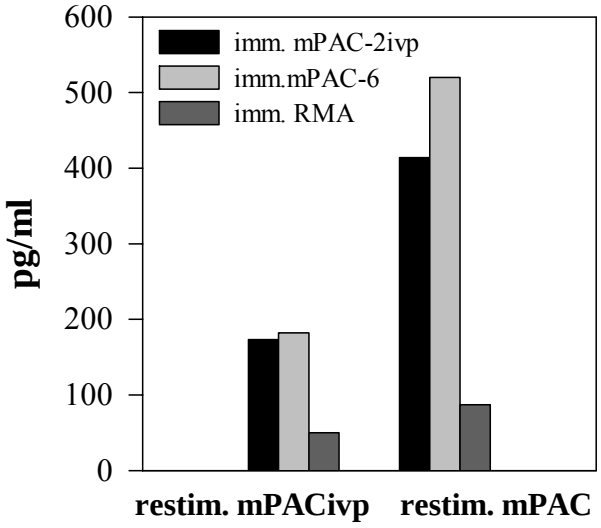

Figure 3.22. IFN-γ detection in supernatants of restimulated lymphocytes. CTL culture supernatants were analysed after 48h for IFN-γ production by ELISA. IFN-γ could only be detected in culture supernatants of lymphocytes derived from mice immunized with mPAC-6 and mPAC-2ivp restimulated with one another.

When mice were immunized with mPAC-6 or mPAC-2ivp and restimulated with mPAC-2ivp, there was at least 3 times more IFN-γ secretion compared to control mice immunized with RMA. When mice were immunized with mPAC-6 or mPAC-2ivp and restimulated with mPAC-6, there was 4 to 5 times more IFN-γ secretion compared to control mice immunized with RMA.

To corroborate these results, a third assay was performed: the same experimental settings were used to analyse IFN-γ secreting lymphocytes by IFN-γ capture assay: After restimulation of lymphocytes the secreted IFN-γ was captured on the cells and subsequently labeled with a second IFN-γ specific antibody. FACS analysis of the cells counterstained with an anti-$CD8^+$ antibody revealed IFN-γ secretion only of $CD8^+$ T cells derived from mice immunized with mPAC-6 or mPAC-2ivp but not with control cell lines (e.g. RMA). Immunization with mPAC-6 resulted in 4% IFN-γ secreting $CD8^+$ T cells after restimulation with mPAC-6 compared to 0,9% IFN-γ positive $CD8^+$ T cells after restimulation with mPAC-2ivp. Similar results were obtained after immunization with mPAC-2ivp: restimulation with mPAC-6 resulted in 9% IFN-γ secreting $CD8^+$ T cells and restimulation with mPAC-2ivp in 0,95%. In contrast to that, no significant numbers of IFN-γ secreting lymphocytes could be detected after immunization with RMA (Fig. 3.23 A).
Furthermore, the IFN-γ production detected by IFN-γ capture assay was confirmed in an independent experiment by intracellular cytokine staining, whereby only IFN-γ produced inside the $CD8^+$ T cells is detected. This explains the quantitative differences in IFN-γ detection between the two methods. Intracellular cytokine staining revealed after immunization with mPAC-6 0,6% IFN-γ secreting $CD8^+$ T cells after restimulation with mPAC-6 compared to 0,3% IFN-γ positive $CD8^+$ T cells after restimulation with mPAC-2ivp. Immunization with mPAC-2ivp resulted in 3% IFN-γ secreting $CD8^+$ T cells after restimulation with mPAC-6 compared to 0,5% IFN-γ positive $CD8^+$ T cells after restimulation with mPAC-2ivp. Lymphocytes derived from RMA-immunized control mice exhibited no significant IFN-γ secretion (Fig. 3.23 B). Looking at these data, it is quite obvious that the original mPAC is not only highly immunogenic *in vivo*, but acts also *in vitro* as the better stimulator compared to mPACivp. In summary, using three different experimental set ups, it can be suggested that mPAC and mPACivp share the same antigens.

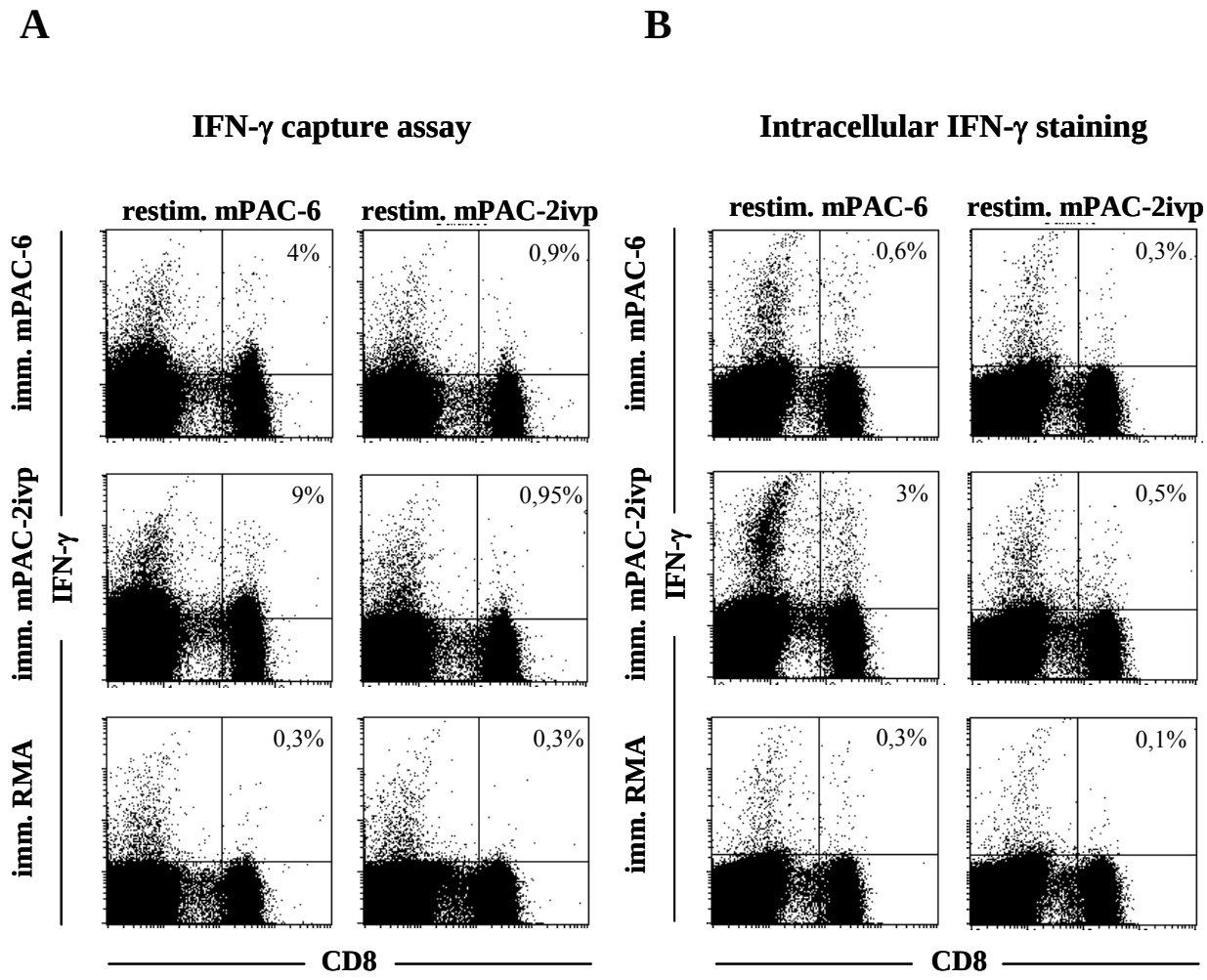

Figure 3.23. Detection of mPAC-6/mPAC2-ivp specific IFN-γ secreting CD8$^+$ T cell by IFN-γ capture assay (A) and by intracellular IFN-γ staining (B). Lymphocytes from mPAC-6 and mPAC-2ivp immunized mice were in *in vitro* restimulated with mPAC-6 or mPAC-2ivp and thereafter analysed for IFN-γ secretion. (A) IFN-γ capture assay revealed a strong IFN-γ secretion of CD8$^+$ T cells derived from mPAC-6 and mPAC-2ivp immunized mice but not from mice immunized with RMA cells. (B) Intracellular IFN-γ staining confirmed IFN-γ production of CD8$^+$ T cells derived from mPAC-6 and mPAC-2ivp immunized mice but not from mice immunized with RMA cells.

3.2.4 T cell infiltration in mPAC-6 and mPAC-2ivp derived tumors

To examine, whether mPAC-2ivp- and mPAC-6 derived tumors were infiltrated with T cells, tumors were explanted at day seven after subcutaneous inoculation. Neither immunohistological nor FACS analysis of tumor infiltrating lymphocytes revealed significant differences concerning tumor infiltrating T cells. In both, the regressing and the progressing tumors, infiltration of $CD4^+$ and $CD8^+$ T cells was found, even though twice as much infiltrating $CD8^+$ cells were identified in mPAC-6 as in mPAC-2ivp derived tumors (6% to 3%) by FACS analysis after Collagenase/Dispase digestion of the tumors (Fig.3.24).

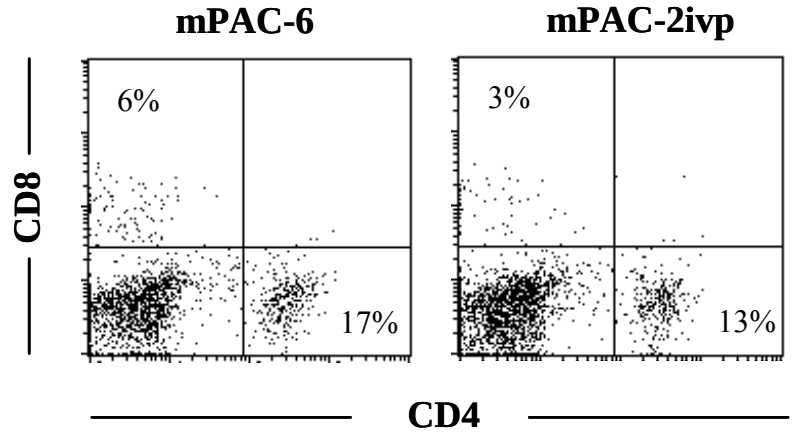

Figure 3.24. mPAC-6- and mPAC-2ivp derived tumors were infiltrated with T cells. Day seven after injection of mPAC-6 and mPAC-2ivp, respectively, tumors were explanted, homogenized and digested and analysed by FACS for $CD4^+$ and $CD8^+$ T cells.

3.2.4.1 Distinct cytokine patterns in mPAC-6 and mPAC-2ivp derived tumors

Since both, mPAC-6 and mPAC-2ivp derived tumors were infiltrated with $CD4^+$ and $CD8^+$ T cells, we wanted to find out, whether the tumor infiltrating lymphocytes in their distinct surroundings exhibit the same functions. Tumors were explanted at day seven after injection, cut into small pieces and cultured over night. Culture supernatants were harvested and analysed for cytokines using ELISA and Cytometric Bead Array (CBA) (Fig. 3.25). CBA revealed that IFN-γ could only be detected in the supernatant of tumors derived from mPAC-6

but in none of the mPAC-2ivp derived tumors. Similarly, also no IL-12 was detected in mPAC-2ivp tumors. On the other hand, TNF-α was found in mPAC-6 as well as in mPAC-2ivp derived tumors at comparable levels. [MCP-I and IL-6 were over detection limit (>5000pg/ml) in all samples tested.] Additional analyses of the supernatants by ELISA confirmed the CBA results for IFN-γ and furthermore revealed similar IL-4 levels in mPAC-6 and mPAC-2ivp derived tumors, but a significant difference in the detected levels of IL-2. Supernatants of explanted mPAC-2ivp derived tumors exhibit on average sixfold higher levels of IL-2 than supernatants of explanted mPAC-6 derived tumors. Furthermore, transforming growth factor (TGF)-β and IL-10, both cytokines which can act as inhibitory cytokines by suppressing anti-tumor immune responses were tested in the supernatants of the tumors by ELISA (TGF-β) and by CBA (IL-10). Neither TGF-β nor IL-10 could be detected

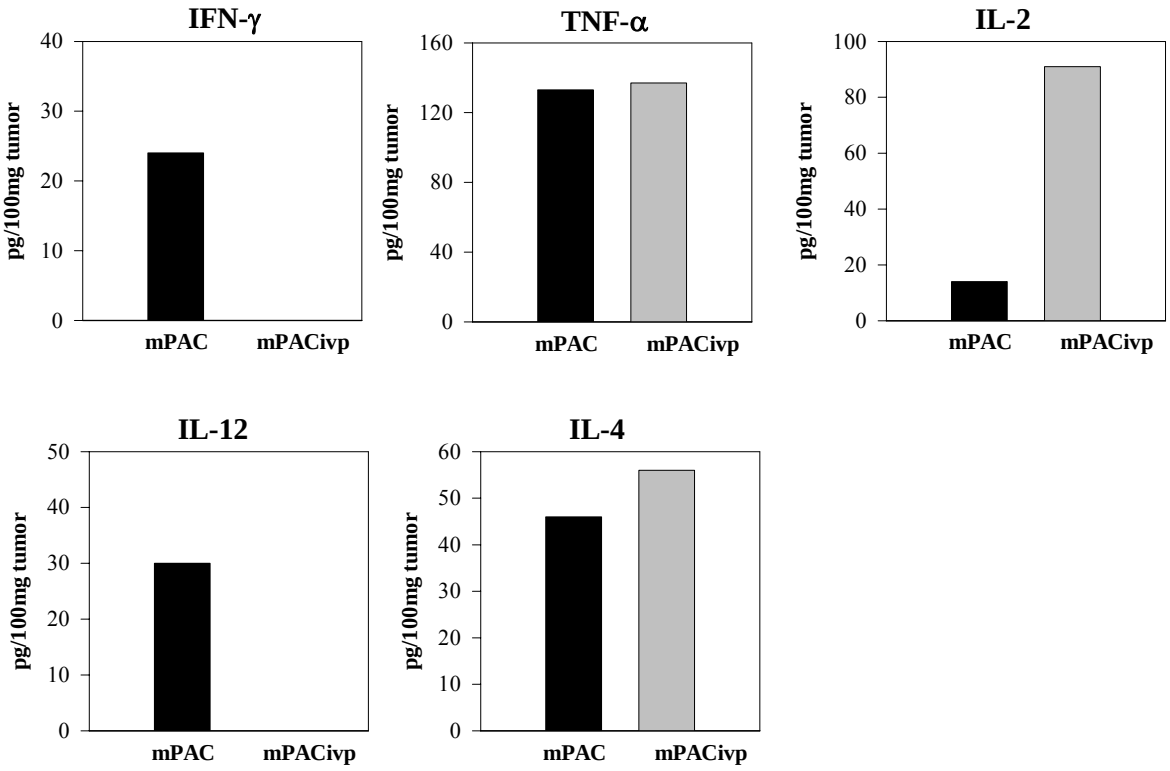

Figure 3.25. Cytokine profiles of mPAC-6 derived compared to mPAC-2ivp derived tumors. Explanted tumors were cut into small pieces and incubated *in vitro*. Culture supernatants of mPAC-6 derived tumors (mPAC, black bars) and of mPAC-2ivp derived tumors (mPACivp, grey bars) were analysed for several cytokines using the cytokine bead array from BD and ELISA. Cytokine quantities are depicted as pg/100mg of the corresponding tumor.

in any of the samples analysed. These data indicate, that the cytokines detected in the supernatant of the immunogenic mPAC-6 reflect mainly an inflammatory milieu. Analysis of an earlier time point after tumor injection (d 5) revealed no IFN-γ production before day seven, but comparable amounts of IL-12 and TNF-α.

3.3 In vivo $CD25^+CD4^+$ T cell depletion

To evaluate, whether regulatory $CD25^+CD4^+$ T cells were involved in dampening tumor-specific immune responses against the escape variant mPAC-2ivp, FACS analysis of lymphocytes derived from draining (inguinal) lymph nodes as well as from the spleen of mPAC-6 or mPAC-2ivp tumor bearing mice for $CD25^+CD4^+$ T cells were performed. C57Bl/6 mice with progressively growing mPAC-2ivp derived tumors did not exhibit elevated numbers of $CD25^+CD4^+$ T cells compared to mice with regressing mPAC-6 derived tumors (Fig. 3.26).

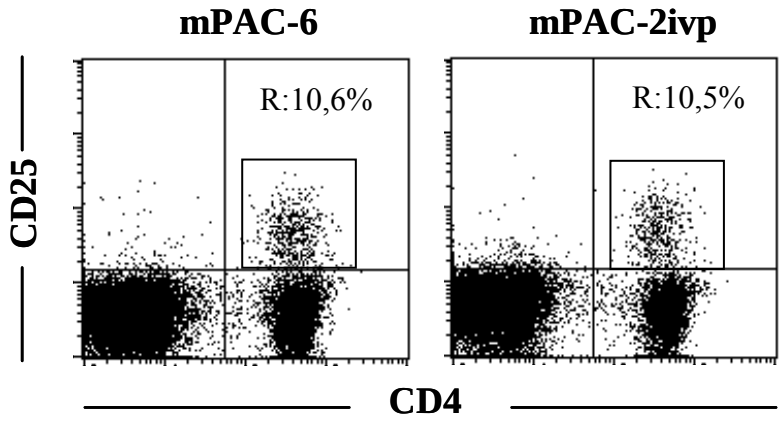

Figure 3.26. Analysis of draining lymphnodes of mPAC-6 and mPAC-2ivp derived tumors for $CD25^+CD4^+$ T cells. Lymphocytes from mPAC-2ivp or mPAC-6 derived tumor bearing mice were isolated and single cell suspensions were stained for the CD25+$CD4^+$ T cells and were analysed by FACS.

In vivo depletion of $CD25^+CD4^+$ T cells before and after mPAC-2ivp inoculation did effectively remove the $CD25^+CD4^+$ T cell subset (Fig. 3.27), but had no influence on the progressive growth of mPAC-2ivp.

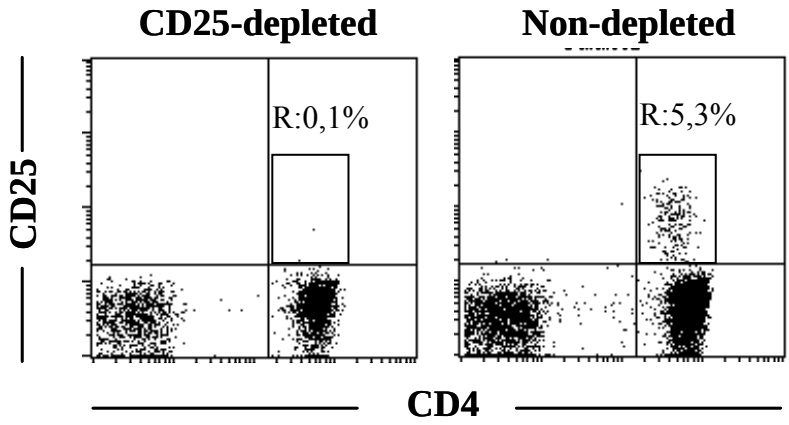

Figure 3.27. *In vivo* depletion of $CD25^+CD4^+$ T cells. $CD25^+CD4^+$ T cells of mPAC-2ivp tumor bearing mice were *in vivo* depleted by the injection of PC61 (for detailed protocol see Material & Methods). Lymphocytes out of whole blood of mice were continuously analysed by FACS for $CD25^+CD4^+$ T cells using an antibody that does not compete with the depleting antibody for binding to $CD25^+CD4^+$ T cells.

3.4 Analysis of the contribution of different components of the immune system to tumor regression

Because mPAC exhibited a regressive phenotype in immune competent wildtype mice but grew progressively to form lethal tumors in RAG1$^{-/-}$ mice, which are deficient of T, B and NKT cells, and with a lag phase also in athymic nu/nu mice, different components of the immune system were analysed.

One possibility to further prove the involvement of T cells in the regression of mPAC-6 derived tumors is to deplete the distinct T cell subsets in immune competent mice *in vivo*. Unfortunately, first experiments to *in vivo* deplete CD8$^+$ and CD4$^+$ T cells did not lead to conclusive results. Although the depletion-antibodies were repeatedly injected according to established, published protocols and T cell depletion was observed in the beginning (Fig.3.28), low numbers of T cells were recovered after tumor inoculation. This was true for both depletion approaches of CD4$^+$ and CD8$^+$ T cells. Due to this, it could not be evaluated whether the tumors regression was due to complete T cell depletion or not a complete depletion.

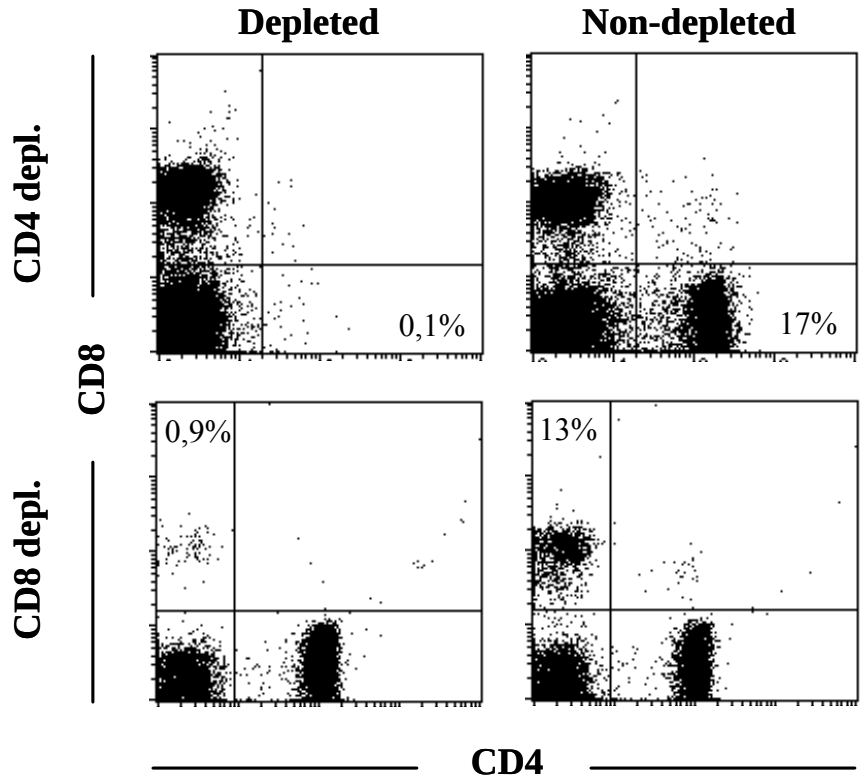

Figure 3.28. *In vivo* depletion of CD4$^+$ and CD8$^+$ T cells. After injection of CD4$^+$- or CD8$^+$-depletion antibody (for detailed protocol see Materials & Methods) splenocytes or lymphocytes out of whole blood were analysed by FACS using antibodies that do not compete with the distinct depletion antibody for binding to CD4$^+$ and CD8$^+$ T cell, respectively.

To check the contribution of NKT cells to mPAC-6 regression, mPAC-6 were s.c. inoculated into $CD1^{-/-}$ mice, which do not have CD1 dependent NKT cells. mPAC showed the same growth kinetics in $CD1^{-/-}$ as in immune competent wildtype mice: The tumor grew during the first ten days and then started to regress. After approximately three weeks no more tumor was palpable. These data suggest that NKT cells were of minor importance in this experimental system.

IFN-γ, as an inflammatory cytokine might play a key role in mPAC regression (Fig. 3.25). For that reason mPAC-6 were injected s.c. into IFN-$\gamma^{-/-}$ mice. Preliminary data indicate that mPAC-6 exhibited similar growth kinetics in IFN-$\gamma^{-/-}$ as in nu/nu mice: after a short growth stagnation the tumor progressed to form lethal tumors after 70 days, leading to the suggestion that IFN-γ plays an important role in the regression of mPAC-6.

The different growth kinetics of mPAC-6 in $RAG1^{-/-}$ compared to nu/nu mice (Fig.3.6) indicate that B cells might also be possible candidates for participating in mPAC regression. Therefore, sera from mPAC tumor bearing immune competent wildtype mice were analysed

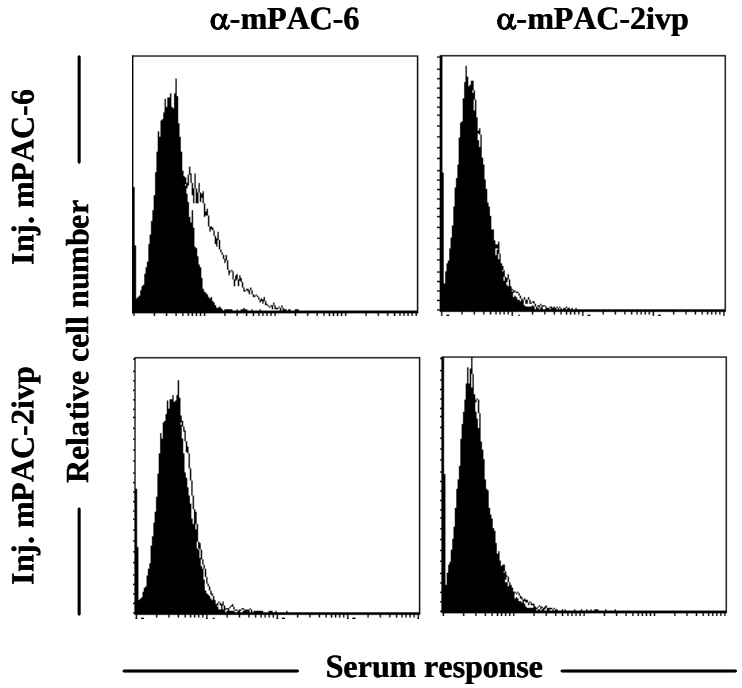

Figure 3.29. mPAC-6 but not mPAC-2ivp induced humoral responses after s.c. injection into C57-Bl/6 mice. Three weeks after injection of mPAC-6, antibodies against mPAC-6 but not against mPAC-2ivp could be identified in sera of mice (1:50 dilution) as shown in histogram overlay. Filled histogram represents analysis of sera taken before injection of mPAC.

for mPAC-specific antibodies. FACS analysis revealed that mPAC-6 induced humoral responses against mPAC-6 but not against mPAC-2ivp, while no antibody response was induced in mPAC-2ivp bearing mice (Fig.3.29). Sera of mice were analysed for mPAC-specific antibodies by FACS analysis.

Analysis of the transplantable mPAC tumor model suggests that tumor growth and regression underlie mechanisms involving T and B cells (and IFN-γ as important inflammatory mediator) that resulted in the selection of an escape variant by decreasing the expression of (an) immune dominant antigen(s) recognized by T and/or B cells.

3.5 Generation of a GM-CSF Vaccine

With the objective of developing a potent vaccine against the progressively growing mPAC-2ivp derived tumors and to further study the possibility that mPAC-6 and the variant mPAC-2ivp share the same antigens, a GM-CSF-expressing variant of mPAC-2ivp was generated. For that purpose, mPAC-2ivp was transduced with supernatant of GM-CSF-retrovirus (CMMP GM-CSF/IRES/GFP) producing cells. Subsequent fluorescence microscopy and FACS analysis revealed GFP expression of mPAC-2ivp/GM-CSF. Analysis of culture supernatants by ELISA showed that mPAC-2ivp/GM-CSF was secreting 2300 ng/1 x 10^6 cells/24h GM-CSF (data not shown). In first vaccination studies, C57Bl/6 mice were immunized with irradiated mPAC-2ivp/GM-CSF and challenged after two weeks with mPAC-2ivp or mPAC-2ivpMHC I^+. These studies showed that the mice were not protected from the outgrowth of mPAC-2ivp by vaccination with mPAC-2ivp/GM-CSF. Control mice and vaccinated mice developed tumors after injection of mPAC-2ivpMHC I^- within the same period of time (45 days) (Fig. 3.30 A). However, the immunization with mPAC-2ivp/GM-CSF resulted in delayed growth of the MHC class I^+ clone of mPAC-2ivp. Non-immunized control mice developed tumors with 4 mm in diameter already after 10 to 18 days and had to be sacrificed after 45 days, whereas GM-CSF vaccinated mice developed tumors of this size not before day 35 and survived until day 60 (Fig. 3.30 B).

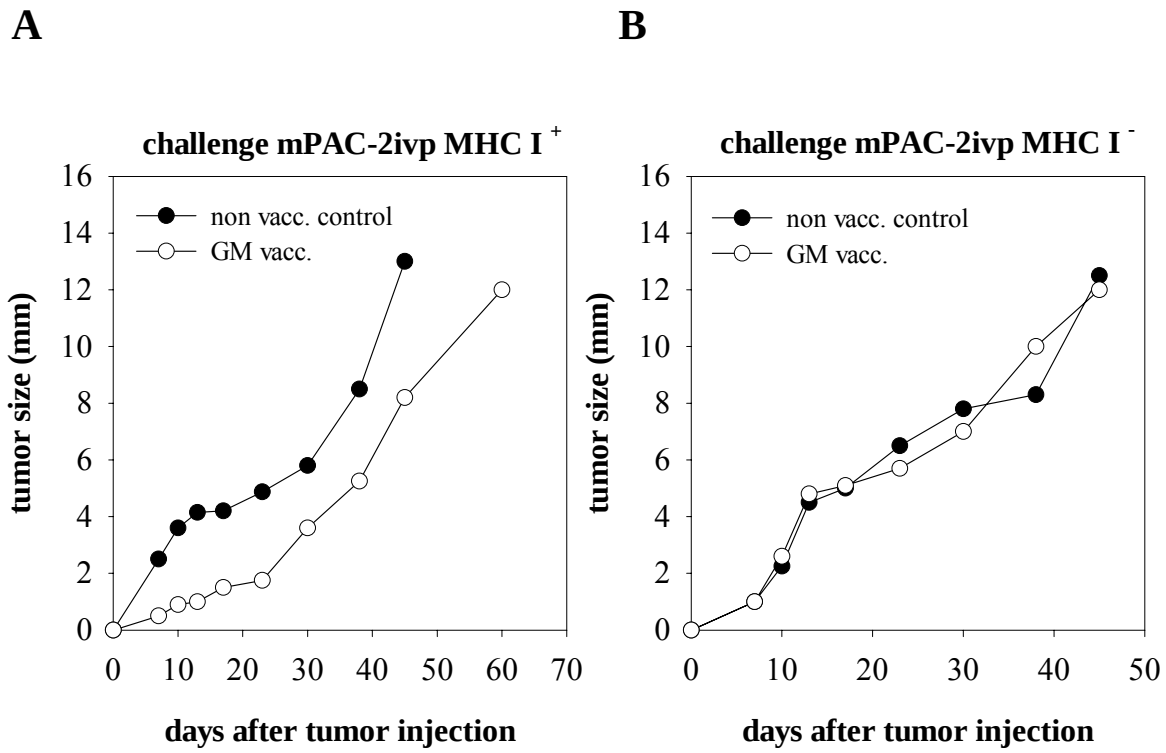

Figure 3.30. Delayed outgrowth of mPAC-2ivp MHC I$^+$ after vaccination with mPAC-2ivp/GM-CSF. Two weeks after immunization with mPAC-2ivp/GM-CSF mice were challenged with mPAC-2ivp MHC I$^+$ or mPAC-2ivp MHC I$^-$ and the tumor growth was monitored.

4 DISCUSSION

4.1 TGF-α Trp53$^{-/-}$ mice as a model for immunological studies on pancreatic cancer

Cancer is a multistep disease involving histological and genetical changes. The extensive studies of the genetic and molecular changes in human tumors provide the basis to understand how a given genetic lesion, or combination of mutations governs specific biological features of the disease during its evolution. Genetically engineered mouse models have contributed extensively to the systematic dissection of these and related issues for a number of cancer types (Macleod and Jacks, 1999; Van Dyke and Jacks, 2002). In contrast to transplantable tumor mouse models, the development of spontaneous tumors in transgenic mouse models is organ specific and the host is conditioned by the physiological events of neoplastic progression and tumorgenesis through various developmental stages. For that reasons information gained from studies using genetically engineered mice that develop autochthonous tumors are more likely transferable to the human settings.

Pancreatic cancer in humans has one of the worst prognosis of all of the gastrointestinal cancers due to late diagnosis and lack of effective treatments. In the case of modelling cancer of the exocrine pancreas, transgenic mouse lines targeting a series of oncogenes to the acinar cell compartment have predominantly produced acinar carcinomas, mixed acinar-ductal tumors, or cystic tumors (Quaife et al., 1987; Ornitz et al., 1987; Sandgren et al., 1991; Grippo et al., 2003; Glasner et al., 1992; Mukherjee et al., 2000; Tevethia et al., 1997; Bardeesy et al., 2002) i.e. most of these models do not reproduce the ductal phenotype of the human disease.

The first described murine tumor progression model for ductal pancreatic cancer that recapitulates the cellular differentiation, the growth characteristics, and the genetic alterations of the human disease is the TGF-α transgenic mouse. In this model, pancreatic cancer develops from premalignant lesions in TGF-α transgenic mice (Sandgren et al., 1990). Overexpression of TGF-α in the pancreas causes a progressive fibrosis and structural transition from acinar cells to tubular complexes (Sandgren et al., 1990; Jhappan et al., 1990; Bockman and Merlino, 1992). After a latency period for more than one year, TGF-α transgenic mice develop malignant pancreatic tumors with a duct-like phenotype. This is further characterized by a loss of acinar markers associated with a gain of duct-specific markers (Wagner et al., 1998). Crossbreeding these mice to p53-deficient mice accelerates

formation of invasive pancreatic carcinoma. Similar to the human disease, genetic events in tumor formation include loss of the wildtype p53 allele in heterozygous p53-deficient mice and inactivation of $p16^{Ink4A}$. Furthermore, constitutive activation of the cyclin-D1-Cdk4 complex and overexpression of EGFR, which induces the Ras/ERK pathway, have been shown (Wagner et al., 2001; Schreiner et al., 2003b; Schreiner et al., 2003a).

Concerning histological and genetical correlations to the human disease, the TGF- α Trp53 deficient mouse has been shown to be an informative animal model for ductal pancreatic cancer and should therefore be useful for immunological studies. Therefore, TGF-α and $Trp53^{-/-}$ mice were bred and crossed on a defined syngenic C57Bl/6 background. In the F2 generation 100% of TGF-α $Trp53^{-/-}$ mice on C57Bl/6 background developed malignant pancreatic tumors within 124 days after birth (Fig. 3.1, 3.2). These results correspond to the results of Wagner *et al*., who performed their studies with TGF-α $Trp53^{-/-}$ mice on a mixed C57Bl/6 x BALB/c background. They showed that all TGF-α $Trp53^{-/-}$ on a mixed C57Bl/6 x BALB/c background developed pancreatic tumors within a comparable period of time (120d) (Wagner et al., 2001). The development of pancreatic tumors in 100% of TGF-a Trp53-/- mice within 124 days makes this tumor model highly feasible for laboratory investigations. The aim of this study was to immunologically characterize these TGF-α $Trp53^{-/-}$ mice to provide a basis for evaluating immunotherapeutic approaches against the autochthonous tumors.

4.2 mPAC induce a strong tumor specific immune response

A cell line derived from the pancreatic tumor of a TGF-α $Trp53^{-/-}$ mouse and thus expressing all potential tumor antigens provides an appropriate tool to perform immunological studies. Although several established murine pancreatic adenocarcinoma cell lines (mPAC) grew progressively *in vitro*, they unexpectedly showed a regressive phenotype after injection into syngenic immune competent C57Bl/6 mice (Fig 3.6). To rule out that the regression of mPAC is due to partial histoincompatibility, mPAC was injected s.c. into the F1 generation of TGF-α x $Trp53^{+/-}$ mice. In these mice mPAC exhibited the same kinetics of initial growth and subsequent regression as observed in C57Bl/6 wildtype mice (data not shown). For that reason, major and minor histocompatibility differences as cause for regression could be

excluded. The spontaneous regression of transplanted syngenic tumors has already been described for several tumor cell lines isolated from spontaneous tumors. In most of the studies this regression has been shown to be T cell dependent, because the tumors grew progressively in nu/nu mice (Gross, 1943; Old et al., 1962; Kripke, 1974; Urban et al., 1982; Martin et al., 1983).

Consistently, we showed that the regression of mPAC-derived tumors in C57Bl/6 mice is an immune regulated phenomenon. This was further defined by the differences seen in growth kinetics of mPAC in nu/nu mice and RAG1$^{-/-}$ mice (Fig. 3.6). In both immune deficient mouse strains mPAC derived tumors grew progressively. But compared to RAG1$^{-/-}$ mice a more delayed tumor growth could be observed in nu/nu mice. Athymic nu/nu mice still produce low but detectable numbers of αβ T cells (Hunig and Bevan, 1980; Ikehara et al., 1984; Maleckar and Sherman, 1987). Therefore, they still can manifest at least some degree of adaptive immunity. Furthermore, in nu/nu mice there are other lymphocyte populations such as NK cells, which are not thymus dependent, as well as γδ T cells and NKT cells that represent two subtypes of T cells, which may develop extrathymically (Hayday, 2000; Kikly and Dennert, 1992; Makino et al., 1993). Gene targeted mice that lack the recombinase activating gene RAG-1 do not have any T, B or NKT cells and exhibit no "leakiness" concerning their T cell deficiency. These data indicate that the regression of mPAC-derived tumors is T cell dependent, but a contribution of other components of the immune system cannot be excluded. For *in vivo* injection those mPAC, which showed the best *in vitro* growth kinetics compared to other murine tumor cell lines such as B16 were chosen (mPAC and B16 showed a comparable *in vitro* growth rate of ~ 1,5 in 24 h). However, the progression of mPAC-derived tumors stagnated shortly after injection into in nu/nu and RAG1-/- mice. Possible reasons for this initial growth lag of the tumors might be interactions of the tumors with the surrounding extracellular matrix leading to inefficient adherence of the tumor cells (Martin et al., 1996).

The involvement of T cells in the rejection of mPAC could be further demonstrated by the detection of T cell responses in immune competent C57Bl/6 mice after inoculation of irradiated mPAC. The induction of cytotoxic responses (Fig. 3.7 A, B) and the observed IFN-γ secretion by CD8$^+$ T cells (Fig. 3.8) lead to the assumption that mPAC express a strong antigen on their surface that is recognized by CD8$^+$ T cells. However, measurement of IFN-γ by intracellular cytokine staining and IFN-γ capture assay has shown that the detected IFN-γ was not only secreted by CD8$^+$ T cells, but in significant amounts by other lymphocytes that

still need to be identified. On the one hand, components of the innate immune system, such as NK and NKT cells can produce IFN-γ and are known to act as effectors in anti-tumor immune responses (Smyth et al., 2000). On the other hand $CD4^+$ TH1 cells, that provide critical help in priming $CD8^+$ cells and are essential in the maintenance of $CD8^+$ T cell effector functions, secrete cytokines such as IL-2 and IFN-γ. Furthermore, some studies have shown that $CD4^+$ T cells are capable of eradication of tumors independently of $CD8^+$ T cells (Greenberg, 1991; Wang, 2001) (Mumberg et al., 1999). Since mPAC do not express MHC class II the direct lysis of mPAC by $CD4^+$ T cells can be excluded in our model, but a significant involvement of CD4 T cells in the regression of mPAC could be suggested. The $CD8^+$ T cell dependency of the cytotoxic response could be further evaluated by using MHC class I positive and MHC class I negative mPAC as targets for CTL assay. NK cells display a subset of inhibitory receptors (e.g. KIR and Ly49) on their surface, which are specific for MHC class I (Colonna and Samaridis, 1995; Mason et al., 1995; Stoneman et al., 1995) and consequently spontaneously kill MHC class I deficient cells. Due to the observed MHC class I dependent lysis of the targets, NK cell as effectors are not very likely.

Most of the transgenic mouse models for cancer overexpress a defined human antigen such as HER-2/neu, CEA or MUC1 (Muller et al., 1988; Eades-Perner et al., 1994; Rowse et al., 1998). Since in our mouse model, TGF-α is overexpressed, TGF-α was suggested to be the potential antigen expressed by mPAC and recognized by $CD8^+$ T cells. For that reason, cytotoxic T cell assays using TGF- α expressing MHC class I^+ targets were performed, but revealed no TGF-α specific lysis (Fig. 3.7 C). In addition, to rule out a possible tolerance of TGF-α transgenic mice towards mPAC expressing the potential antigen TGF-α, growth kinetics of mPAC after s.c. injection into TGF-α transgenic mice were analysed, mPAC derived tumors show the same regressive phenotype in TGF-α transgenic mice as in non-transgenic wildtype control mice. These data suggest, that TGF-α can be excluded as the potential antigen.

To determine whether existing CTLs can successfully access the mPAC derived tumors, tumor sections were stained for infiltrating lymphocytes and in addition, the tumor infiltrating lymphocytes were analysed by FACS. mPAC derived tumors were highly infiltrated by $CD4^+$ and $CD8^+$ T cells (Fig. 3.9). This was accompanied by the secretion of IFN-γ, IL-12 and TNF-α detected in culture supernatants of the explanted tumors (Fig. 3.14). The detected cytokine profile underlines the inflammatory response induced by mPAC inoculation. In addition to the

disitinct T cell responses induced by mPAC, peripheral humoral responses against mPAC was found in the sera of immune competent mice after regression of mPAC derived tumors. Thus far our data suggest, that the highly immunogenic cell line mPAC represents a powerful tool to analyse immune responses in TGF-α Trp53$^{-/-}$ mice.

4.3 Detection of mPAC specific immune responses in TGF-α Trp53$^{-/-}$ mice

It has been shown that HER-2/neu mice are immunological tolerant against the antigen or against cell lines transfected with the antigen (Reilly et al., 2000). In other transgenic mouse lines, such as MET mice, no immunological tolerance could be observed (Mukherjee et al., 2000). Interestingly, spontaneous immune responses could be detected in TGF-α Trp53$^{-/-}$ mice. This spontaneous immune response was characterized by CD8^{+} T cells that secreted IFN-γ after *in vitro* restimulation with mPAC (Fig. 3.13), but no cytotoxic activity against mPAC was detected. mPAC specific IFN-γ secretion could only be detected in 12-week-old TGF-α Trp53$^{-/-}$ mice but not in 6-week-old TGF-α Trp53$^{-/-}$ mice nor in age-matched TGF-α Trp53$^{+/-}$control mice. These data suggest a relation between tumor progression and the induction of an immune response. Only those mice that already developed malignant pancreatic adenocarcinoma exhibited CD8^{+} T cell responses. Furthermore, spontaneous antibody responses against mPAC could be detected in sera of TGF-α Trp53$^{-/-}$ mice, which increased with progression of the autochthonous tumors (Fig. 3.15). The finding that *in vitro* restimulation of CD8^{+} T cells derived from 12-week-old TGFα Trp53$^{-/-}$ mice triggers IFN-γ release, but not specific lysis of the tumor cells *in vitro*, leads to the assumption that the CD8^{+} T cells were only partially activated. However, it was possible to induce CTL responses in TGFα Trp53$^{-/-}$ mice after vaccination, indicating that the CD8^{+} T cells have no general functional defect (data not shown). Additionally, independent of their age, TGFα Trp53$^{-/-}$ mice are able to reject transplanted mPAC derived tumors in the same manner as wildtype mice (data not shown). To further analyse the T cell function in TGFα Trp53$^{-/-}$ mice, culture supernatants of pancreatic tumors were analysed for cytokines. In sharp contrast to mPAC derived tumors, no significant levels of inflammatory cytokines could be detected in autochthonous tumors (Fig. 3.14). This finding was not unexpected, since tumor infiltrating lymphocytes were not found in autochthonous tumors at any stage of tumor development.

Double transgenic MET mice represent a transgenic mouse model for pancreatic cancer that has already been immunologically characterized. MET mice, obtained by crossing MUC1.tg mice with ET mice, which express a truncated form of Tag under the control of the elastase promotor, develop spontaneously MUC1-expressing acinar cell carcinoma in the pancreas (Mukherjee et al., 2000). It has been decribed that MUC1 transgenic mice develop B and T cell compartment tolerance and are refractory to immunization with the protein encoded by the trangene (Rowse et al., 1998), but non-immunized MET mice develop MUC1-specific CTL-precursor activity. Lysis of MUC1 expressing target cells increased with tumor progression. In addition, low, but detectable levels of circulating antibodies to MUC1 could be detected in some of the older tumor bearing mice (Mukherjee et al., 2000). These data are in strong correlation with the immune responses detected in TGF-α Trp53$^{-/-}$ mice. In both tumor models spontaneous cellular and humoral immune responses increasing with tumor burden could be observed, but although no cytotoxic lysis of mPAC could be detected *in vitro*, TGF-α Trp53-/- mice were able to reject mPAC derived tumors *in vivo*.

In the MET mouse model, the adoptive transfer of CD8$^+$ T cell lines established from MET mice resulted in the complete rejection of transplanted MUC1 expressing tumors in MUC1 mice, but the direct *in vivo* function of these CD8$^+$ T cells in MET mice was not shown. Thus, a direct comparison of the function of the tumor specific CD8$^+$ T cells between these two transgenic mouse models for spontaneous pancreatic tumors is not possible. However, the significance of immunological studies performed with a mouse model for acinar carcinoma of the pancreas that does not mimic the human disease such as the MET mouse remains questionable. In another spontaneous tumor mouse model, the immune response against a transplantable tumor cell line was directly evaluated in transgenic mice bearing autochthonous tumors. CEA transgenic mice (tg) have been shown to be immunologically tolerant to CEA (Eades-Perner et al., 1994; Clarke et al., 1998). Because CEA.tg mice do not develop spontaneous tumors, CEA.tg mice were crossed with Apc knock out mice. The recently described Apc1638N/CEA mice develop spontaneous tumors expressing CEA in the GI tract and exhibit humoral but not T-cell tolerance to CEA. Furthermore, Apc1638N/CEA mice showed delayed growth of transplantable CEA-expressing tumors, as compared to CEA.tg mice, but the tumors grew faster in Apc1638N/CEA mice than in non-transgenic wildtype mice (Horig et al., 2001; Kantor et al., 1992).

This is in contrast to the regression of mPAC in TGF-α Trp53$^{-/-}$ mice that we have shown to be comparable to the regression of mPAC in wildtype mice. The finding that TGF-α Trp53$^{-/-}$

are able to reject the transplanted tumor cell line expressing the potential antigen might indicate that in this mouse model for ductal pancreatic cancer the induction of an effective immune response by vaccination might be more effective than in the models described above.

In addition, in these spontaneous tumor models the existing CTL were unable to eradicate the autochthomous tumors, which resembles the situation for most cancer patients. It has been shown in humans and mice that immunogenic tumors can coexist with anti-tumor lymphocytes without any impact on tumor progression (Lee et al., 1999; Staveley-O'Carroll et al., 1998). Many different reports have suggested that progressing tumors have elaborated means of escaping an apparently effective MHC class I restricted immune response (Garrido et al., 1997; Algarra et al., 1997; Cabrera et al., 1996; Hicklin et al., 1998). Others have found that the CTL response occurs too late to be effective against the established tumor (Toes et al., 1996). Another reason for the escape of the autochthonous tumor might be that the induced T cells are low avidity T cells (Kawai and Ohashi, 1995; Hausmann et al., 1999). Defective lymphocyte homing to the tumor could be another explanation for the failure of CTLs to control tumor growth (Onrust et al., 1996). Alternatively, efficient anti-tumor CTL acticity may initially be induced but then declines (Speiser et al., 1997). Other reports demonstrate tumor escape is likely due to limited degree of cross-presentation in the regional lymph nodes, which would result in insufficient numbers of activated tumor-specific T cells to protect the host from tumor growth (Nguyen et al., 2002) or due to the overwhelming ratio of antigen-bearing tumor cells relative to tumor-specific CTL (Hanson et al., 2000; Nelson et al., 2001). Our data imply that the CTL do not enter the tumor matrix and are therefore not able to prevent tumor growth. An approach to address this issue in our tumor model is to adoptively transfer lymphocytes derived from mPAC primed immune competent mice into TGF-α $Trp53^{-/-}$ mice. These lymphocytes should be labelled in a way that they can be easily recovered in the TGF-α $Trp53^{-/-}$ mice to obtain conclusive results concerning failure in lymphocyte homing to the autochthonous tumors. However, preliminary results from one experiment using TGF-α $Trp53^{-/-}$ mice, demonstrated that the adoptively transferred lymphocytes did not invade into the autochthonous tumors. Further experiments are needed to complete these studies. Nevertheless, TGF-α $Trp53^{-/-}$ mice represent an excellent model to investigate phenomena of immune escape.

4.4 Selection of the variant mPAC-2ivp: insights into mechanisms of tumor growth and regression

We have shown that when mPAC derived tumors were injected into immune competent mice, they regressed within 21 days after injection, but grew progressively, even though with a short delay, when injected into RAG1$^{-/-}$ mice. Therefore, these pancreatic tumor cell lines consist of potentially malignant cells that can be used to study the relative importance of different immune surveillance mechanisms that may function in immune competent animals. As a consequence of malignant transformation, malignant cells acquire genetic instability and within the tumor phenotypic variants may arise that exhibit selective growth advantages (Nowell, 1976). These variants can escape immunological or other homeostatic control mechanisms of the host and become the precursors of a newly emerging subpoplation that now become dominant (Urban et al., 1982). The tumor variant mPAC-2ivp did arise spontaneously in a normal immune competent C57Bl/6 mouse from the regressive growing pancreatic adenocarcinoma cell line mPAC. Injection of mPAC-2ivp cells produced progressively growing tumors in 100% of the immune competent syngenic mice (Fig. 3.16), thereby demonstrating the heritable nature of the progressive growth of the newly isolated tumor mPAC-2ivp. The establishment of the new cell line mPAC-2ivp provides the opportunity to analyse possible escape mechanism of solid tumors in a fast growing transplantation tumor model.

The first issue to address was to analyse the influence of MHC class expression on progressive tumor growth of mPAC-2ivp. The MHC class I $^{-}$ cell line mPAC-2ivp and its MHC class I $^{+}$ clone exhibited comparable *in vivo* growth kinetics after s.c. injection into normal mice (Fig. 3.18). Independent of their status of MHC class I expression, all tumors grew progressively to form lethal tumors, indicating that loss or down-regulation of MHC class I seems not to be the mechanism that led to the immune escape of mPAC-2ivp.

To examine whether mPAC-6 and the variant mPAC-2ivp share the same potent CTL antigen, *in vivo* and *in vitro* experiments were performed. Triggering the immune system of C57Bl/6 mice by the s.c. inoculation of mPAC-6 did lead to impaired growth of a second challenge with mPAC-6 (Fig. 3.19 A), emphasizing once again the immunogenic phenotype of the original cell line mPAC-6, but did not prevent the progressive growth of the variant mPAC-2ivp (Fig.3.19 B). This immunological unresponsiveness of the mPAC-6-primed mice

towards challenge with mPAC-2ivp suggests that mPAC-6 and mPAC-2ivp do not express the same antigens on their surface. Moreover, mice already bearing the progressive growing variant mPAC-2ivp did not tolerate the growth of mPAC-6 (Fig. 3.19 C). Other studies have shown with comparable experiments that their regressive and progressive variants of the same tumor are immunologically related (Caignard et al., 1985).

Analysing the induction of immune responses after inoculation of mPAC-6 or mPAC-2ivp led to different results. CTL assays performed with mPAC-6 or mPAC-2ivp as restimulators and targets revealed cross-reactivity of the two variants. A mutual and clearly MHC class I dependent cytotoxic activity could be shown for almost all conditions analysed (Fig. 3.21, Tab. 3.2). The analysis of CTL supernatants showed that only lymphocytes derived from mice immunized with mPAC-6 or mPAC-2ivp and not with irrelevant tumor cell lines secrete IFN-γ upon restimulation with one of the variants (Fig. 3.22). Measurement of IFN-γ either by intracellular cytokine staining or by capture assay have shown that a significant amount of IFN-γ is secreted by $CD8^+$ T cells (Fig. 3.23). This indicates that mPAC-6 and the variant mPAC-2ivp can induce *in vivo* cellular responses against the same antigens. Both, mPAC-6 and mPAC-2ivp could be used as mutual restimulators and targets in CTL. The regressive phenotype of mPAC-6 in immune competent mice showed that mPAC-6 is highly immunogenic *in vivo*. This is supported by the CTL data, showing that in mice immunized with mPAC-6 stronger immune responses are induced as in those mice immunized with mPAC-2ivp. Furthermore, the three different ways of analysing IFN-γ secretion (ELISA, IFN-γ capture assay, intracellular staining) all reveal that mPAC-6 acts also as the better stimulator *in vitro*. These results suggest that the escape variant mPAC-2ivp has not lost but down-regulated the expression of the immune dominant antigen, otherwise lymphocytes derived from mice immunized with mPAC-2ivp would not recognize mPAC-6 *in vitro*. In human cancers decreased expression of tumor antigens have also been described to be associated with disease progression (de Vries et al., 1997). A decreased expression of the potential antigen in mPAC-2ivp might not be sufficient for tumor recognition and subsequent rejection but the antigen could be effectively cross-presented to $CD8^+$ T cells by APCs to induce the immune response detected in CTL and IFN-γ assays. This could also explain the results of the injection of mPAC-6 and mPAC-2ivp into the same animal (Fig. 3.19). However, the data cannot rule out the possibility that the antigen recognized in CTL on mPAC-2ivp is not the same as the potential immunodominant antigen that is recognized *in vivo* and leads to the rejection of mPAC-6. It has been shown in the extensive studies by Schreiber *et al*. that

progressively growing variants can lose their original tumor-specific transplantation antigen but can thereby retain their sensitivity to cross-reactive CTL-mediated killing *in vitro*, implicating that the CTL-recognized antigens detected *in vitro* are not necessarily the targets for rejection *in vivo* (Urban et al., 1982; Ward et al., 1990; Mumberg et al., 1996). It has been also reported that lack of cross-protection is not necessarily due to lack of a shared tumor antigen (Ramarathinam et al., 1995). More detailed studies are required to address this issue.

To examine, whether the induction of CTLs by the original mPAC-6 and the escape variant mPAC-2ivp leads to infiltration of T cells into tumors, immunohistological and FACS analysis of tumor infiltrating lymphocytes were performed. Surprisingly, infiltration of $CD4^+$ and $CD8^+$ T cells was found in mPAC-6 as well as in mPAC-2ivp derived tumors, even though twice as much infiltrating $CD8^+$ cells were identified in mPAC-6 as in mPAC-2ivp derived tumors (6% to 3% [Fig. 3.24]). Tumors can be infiltrated by T cells but the T cells in the tumor might be impaired in their function as effector cells. Therefore, supernatants of explanted mPAC-2ivp derived tumors were analysed in comparison to mPAC-6 derived tumors for cytokine secretion. Looking at the cytokine profile of the distinct tumors revealed a significant difference: One of the most important mediators for inflammation, IFN-γ could only be detected in the supernatants of mPAC-6 derived tumors and in none of the progressive growing mPAC-2ivp derived tumors. In accordance with this, IL-12, known to induce IFN-γ secretion, could also only be detected in mPAC-6 tumors. However, TNF-α and IL-4 were found in both tumor types at comparable levels. The only cytokine detected at significant higher levels in mPAc-2ivp derived tumors was IL-2 (Fig. 3.25).

Mice deficient for IFN-γ or IFN-γ receptor have been shown to be more susceptible to the formation of tumors (Kaplan et al., 1998; Street et al., 2002; Qin et al., 2003; Nakajima et al., 2001). Because significant levels of IFN-γ were detected in mPAC-6 derived tumors but not in the progressive mPAC-2ivp derived or autochthonous tumors, mPAC-6 was injected into IFN-$\gamma^{-/-}$ mice. Preliminary experiments point out that IFN-γ plays an important role in the regression of mPAC-6, because in IFN-$\gamma^{-/-}$ mice mPAC-6 derived tumors showed progressive growth kinetics comparable to nu/nu mice.

The prevalence of IL-2 in mPAC-2ivp derived tumors might be an evidence for the involvement of $CD25^+CD4^+$ regulatory T cells in dampening tumor-specific immune responses against the escape variant mPAC-2ivp (Liyanage et al., 2002; Woo et al., 2001;

Woo et al., 2002). Compared to mPAC-6 tumor bearing mice, mPAC-2ivp tumor bearing mice did not exhibit elevated numbers of $CD25^+CD4^+$ regulatory T cells (Fig. 3.26). To evaluate a possible functional difference between the $CD25^+CD4^+$ regulatory T cells populations in the distinct mice, $CD25^+CD4^+$ regulatory T cells were depleted in mPAC-2ivp tumor bearing mice (Fig. 3.27), but did not result in spontaneous tumor-specific CTL and NK cell cytotoxicity and subsequent tumor eradication as it has been shown for other tumor models (Shimizu et al., 1999; Onizuka et al., 1999). The production of inhibitory cytokines as mechanism for tumor escape from immunologic destruction could be excluded, because neither TGF-β nor IL-10 could be detected in the supernatant of tumors isolated from mPAC-2ivp bearing mice.

In summary, the loss of immunogenicity of the variant mPAC-2ivp most likely seems be the result of decreased expression of the immune dominant antigen. This idea was strongly supported by the fact that only mPAC-6 could induce humoral responses against mPAC-6 but not against mPAC-2ivp, while no antibody response at all is induced in mPAC-2ivp bearing mice (Fig. 3.29). That antibodies specific for mPAC-6 did not bind to mPAC-2ivp leads to the suggestion that mPAC-2ivp has lost the expression of a certain surface antigen. This appears to be constistent with the different growth kinetics of mPAC-6 seen in $RAG1^{-/-}$ compared to nu/nu mice (Fig. 3.6), indicating that B cells might also be possible candidates for participating in mPAC regression and emphasizing once again the role of CD4 cells in this model.

Antibody-induced down-modulation of the target antigen has been reported for a number of cell surface receptors and represents a relatively rapid event occurring immediately after antibody exposure (Sorkin and Waters, 1993). Vesicles internalising the receptors fuse with lysosomes, which results in increased degradation and reduced steady state levels of the receptor. This has already been shown in a number of studies on the human epidermal growth factor receptor HER-2/neu or ErbB-2. The loss of HER-2/neu expression by down-modulation significantly inhibits growth and causes reversal of the transformed phenotype of the tumor (Drebin et al., 1985; Klapper et al., 1997; Baselga, 2001). It has been shown in TGF-α transgenic mice that overexpression of TGF-α results in up-regulation of its receptor EGFR (Wagner et al., 1998). Activation of epidermal growth factor receptor results in potent signals of cell proliferation, survival, invasion, and angiogenisis (Olayioye et al., 2000). Considering the hypothesis that mPAC regresses because of antibody-induced down-

modulation of EGFR it would not explain why the variant mPAC-2ivp that potentially has lost the surface expression of EGFR is still surviving and progressively growing.

Another reason why mPAC regression cannot solely be dependent on B cells is the progressive growth of mPAC derived tumors in nu/nu mice that are described to exhibit significant B cell function. Thus, the regression of mPAC and moreover the escape of mPAC-2ivp are more likely due to a interaction of B and T cells. Similar to that, in transgenic mouse models for mammary adenocarcinoma overexpressing HER-2/neu conflicting results exists with regard to the role of antibodies/B cell versus T cells in tumor rejection in HER-2/neu based vaccination approaches. On the one hand, the need for cooperation between humoral and cellular immunity was reported, on the other hand specific antibodies generated by different vaccination protocols were found to correlate with immunity against spontaneous tumors (Reilly et al., 2001; Wolpoe et al., 2003; Nanni et al., 2001; De Giovanni et al., 2004).

To further evaluate the mechanisms leading to growth and regression of mPAC-2ivp and mPAC, respectively, different components of the innate immune system were analysed. Therefore, mPAC-6 was injected into NKT cell deficient $CD1^{-/-}$ mice. mPAC-6 showed the same regressive phenotype in $CD1^{-/-}$ mice as observed in immune competent wildtype mice (data not shown). For that reason, NKT cells seem not to be the key component of the immune system leading to the regression of mPAC. As an important actor in antibody-dependent cell mediated cytotoxicity (ADCC), NK cells recognize IgG antibody coated cells via their FcγRIII receptor (Ravetch and Perussia, 1989; Lanier et al., 1989; Perussia et al., 1984). Compared to $RAG1^{-/-}$ mice a more delayed tumor growth could be observed in nu/nu mice after injection of the original mPAC (Fig 3.6). Nu/nu exhibit significant B cell functions and also NK cells are unaffected in nu/nu mice. To rule out, whether ADCC might contribute to the regression of mPAC and therefore might explain the difference seen between the growth of mPAC in nu/nu compared to $RAG1^{-/-}$ mice, mPAC was injected into SCID.beige mice that lack T, B, NKT and NK cells. mPAC showed comparable growth kinetics in SCID.beige mice as in $RAG1^{-/-}$ mice, indicating that NK cells seem not to be very likely the main components of the immune system leading to the regression of mPAC. *In vivo* depletion of NK cells should be performed to further evaluate the contribution of NK cells to tumor regression.

In conclusion, analysis of the transplantable mPAC tumor model suggests that tumor growth and regression underlie mechanisms involving T and B cells (and IFN-γ as important inflammatory mediator). These mechanisms are suggested to result in the selection of an escape variant by decreasing the expression of (an) immune dominant antigen(s) recognized by T and/or B cells. Further studies should help to clarify the role of the distinct lymphocyte populations. *In vivo* depletion of single or combined lymphocyte subsets represent one possible route. Another approach to identify the potential immunodominant antigen might be searching for decreased expressed gene products in mPACivp compared with mPAC by gene expression profiling.

The results obtained from our extensive study on mPAC-6 and the variant mPAC-2ivp should be conclusive to understand the mechanisms leading to the escape of the autochthonous tumors in TGF-α $Trp53^{-/-}$ mice from immune destruction. Decreased antigen expression might also be the mechanism leading to the escape of the solid pancreatic tumors as proposed for tumors derived from the progressively growing variant mPAC-2ivp. The production of inhibitory cytokines such as IL-10 could be excluded for the transplanted as well as for the autochthonous tumors as a possible mechanism, but in contrast to tumors derived from mPAC-2ivp, the autochthonous tumors were not infiltrated by neither $CD4^+$ nor $CD8^+$ T cells. This observation suggests that the tumor stroma and the lymphocytes in the TGF-α $Trp53^{-/-}$ mice need to be further investigated (see paragraph 4.3). In addition, we did not find quantitative differences concerning $CD25^+CD4^+$ T cell populations in TGF-α $Trp53^{-/-}$ mice compared to wildtype littermate control mice. *In vivo* depletion of this lymphocyte subset should give us further information, whether in this mouse model for pancreatic adenocarcinoma $CD25^+CD4^+$ T cells play an important role in suppression of an effective anti-tumor response.

Furthermore, immunotherapy approaches could be established and primarily evaluated in the transplantable tumor model before they would be transferred to the spontaneous tumor model. Studies in other spontaneous tumor models have shown that in most models only a very extensive immunization protocol led to reduced tumor burden and significantly improved long term survival. Recently published promising approaches include combination of a poxvirus-based vaccine with a triad of costimulatory molecules (TRICOM) + GM-CSF or in combination with an anti-inflammatory drug in CEA.Tg/min mice (Greiner et al., 2002;

Zeytin et al., 2004), vaccination with dendritic cells fused with tumor cells in MTT mice (Xia et al., 2003; Chen et al., 2003) and in APC mice (Iinuma et al., 2004), combination of T-cell therapy and irradiation or protein vaccination with CpG-ODN as adjuvant in RIP1-Tag5 mice (Ganss et al., 2002; Garbi et al., 2004) and IL-12-engineered allogenic cell vaccines in HER-2/neu.tg mice (De Giovanni et al., 2004).

Our data provide the first insights into tumor specific cellular and humoral immune responses induced in a novel murine model for ductal pancreatic adenocarcinoma. The careful evaluation of the different vaccination studies mentioned above should help us to develop suitable immune therapeutic approaches against the autochthonous tumors in TGF-α $Trp53^{-/-}$ mice.

5 SUMMARY

Pancreatic adenocarcinoma is a fatal disease. Immunotherapy could prove to be a valuable therapeutic option, however, until today no appropriate animal model has been available to test this. TGF-α Trp53$^{-/-}$ mice develop pancreatic adenocarcinoma within 124 days after birth. Histological examination of these tumors reveal typical structures of ductal pancreatic adenocarcinoma with irregular cellular morphology mimicking the human disease. In this study, TGF-α Trp53$^{-/-}$ mice were crossed on C57Bl/6 background and it was demonstrated that these mice provide a suitable model to test immunotherapeutic approaches against ductal pancreatic adenocarcinoma.

Several murine pancreatic adenocarcinoma cell lines (mPAC) derived from tumors of TGF-α Trp53$^{-/-}$ mice were generated. These cell lines showed a regressive phenotype after injection into C57Bl/6 mice. The regression was immune mediated, since after subcutaneous inoculation into immune deficient mice, mPAC grew slow but progressively to form lethal tumors. The more delayed growth of mPAC-6 in nu/nu mice compared to the growth in RAG1$^{-/-}$ indicates the contribution of T and B cells to tumor regression. Further studies demonstrated that inoculation of immune competent C57Bl/6 mice with mPAC-6 induced tumor-specific humoral and CTL responses. The shown cytotoxic responses were consistent with the detected IFN-γ secretion of CD8^{+} T cells.

TGF-α Trp53$^{-/-}$ mice with sponataneous ductal pancreatic adenocarcinoma were also analysed. These mice developed tumor-specific cellular and humoral immune responses which were only found in tumor bearing mice but not in mice with premalignant lesions. Despite the spontaneous tumor-specific immune responses, no tumor infiltrating lymphocytes could be detected at any stage of tumor development in TGF-α Trp53$^{-/-}$ mice.

Finally, an *in vivo* passaged variant mPAC-2ivp was generated, which showed reproducible progressive *in vivo* growth in immune competent C57Bl/6 mice. Although mPAC-2ivp induced no humoral tumor-specific responses, a cross-reactive cytotoxicity with mPAC-6 could be detected, indicating that the escape variant mPAC-2ivp has not lost but might exhibit decreased expression of immune dominant antigen(s). Furthermore, our data suggest that neither loss of MHC class I expression, nor the secretion of inhibitory cytokines or the presence of regulatory T cells are the mechanisms that have led to the escape of mPAC-2ivp.

These extensive studies on mice with spontaneously developing pancreatic adenocarcinoma, on the spontaneous tumor-specific immune responses as well as on the transplantable mPAC tumor model provide a powerful tool to characterize the mechanisms leading to the escape of the autochthonous tumors and furthermore to develop suitable immunotherapeutic approaches in TGF-α $Trp53^{-/-}$ mice against murine pancreatic adenocarcinoma.

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ABBREVIATIONS

ADCC	Antibody-dependent cell mediated cytotoxicity
AICD	Activation-induced cell death
APC	Antigen presenting cell
Apc	Adenomatous polyposis coli
bp	Base pairs
CBA	Cytometric bead array
CD	Cluster of differentiation
CEA	Carcinoembryonic antigen
CK	Cytokeratin
CpG	Cytosine-phosphorothioate-guanine
cpm	counts per minute
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EL	Elastase
ELISA	Enzyme linked immunosorbent assay
FACS	Fluorescence activated cell sorter
GM-CSF	Granulocyte-macrophage-colony stimulating factor
GFP	Green fluorescent protein
HER	Human epidermal growth factor receptor
hGH	Human growth hormone
HLA	Human leukocyte antigen
HSP	Heat-shock protein
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
i.p.	intraperitoneal
ivp	*in vivo* passaged
MCA	methylencholanthrene
MHC	Major histocompatibility complex

MMTV	Mouse mammary tumor virus
mPAC	Murine pancreatic adenocarcinoma cell line
MUC	Mucin
NK	Natural killer
OD	Optical density
ODN	Oligodeoxynucleotides
o.n.	Over night
PanINs	Pancreatic intraepithelial neoplasia
PCR	Polymerase chain reaction
RAG	Recombinase activating gene
RIP	Rat insulin promoter
RT	Reverse transcription
RT-PCR	Reverse transcription dependent polymerase chain reaction
s.c.	subcutaneous
SCID	Severe combined immunodeficiency
TGF	Transforming growth factor
TH	T helper
TILs	Tumor infiltrating lymphocytes
Tg	Trangene, transgenic
TNF	Tumor necrosis factor
Trp	Transformation related protein
wt	wildtype

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